IMMUNITY TO MALARIA AND VACCINE STRATEGIES

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IMMUNITY TO MALARIA AND VACCINE STRATEGIES

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Malaria, caused by infection with protozoan parasites belonging to the genus *Plasmodium*, is a highly prevalent and lethal infectious disease, responsible for 435,000 deaths in 2017. Optimism that malaria was gradually being controlled and eliminated has been tempered by recent evidence that malaria control measures are beginning to stall and that *Plasmodium* parasites are developing resistance to front-line anti-malarial drugs. An important milestone has been the recent development of a malaria vaccine (Mosquirix) for use in humans, the very first against a parasitic infection. Unfortunately, this vaccine has modest and short-lived efficacy, with vaccinated individuals possibly being at increased risk of severe malarial disease when protection wanes. Thus, to define new ways to combat malaria, there remains an urgent requirement to identify the immune mechanisms that promote resistance to malarial disease and to understand why these so often fail.

The review and primary research articles in this Research Topic illustrate the breadth of research performed worldwide aimed to understand the biology of the *Plasmodium* parasite, the roles of the various cell types that act within the immune response against the parasite, and the parasitological and immunological basis of severe malarial disease. The articles in section 1 exemplify the different vaccination strategies being developed and tested by the research community in the fight against malaria. The articles in section 2 review important overarching aspects of malaria immunology and the use of models to study human malaria. The articles in section 3 describe the ways through which the *Plasmodium* parasite is initially recognised by the immune system during infection, how the parasite can directly impact this critical event to restrict anti-Plasmodial immunity, and resolve the roles of key innate cell populations, such as dendritic cells, in coordinating malarial immunity. The articles in sections 4-6 outline the roles T and B cell populations play during malaria, highlighting the activation, diversification and regulation of these crucial cell types during malaria, and discuss some of the reasons adaptive immunity to malaria is often considered so poor compared with other diseases. The articles in section 7 provide up to date information on the pathogenesis of cerebral malaria, bridging our understanding of the syndrome in humans with information learned from animal models. Overall, the articles in this research, many of which are published by leaders in the malaria field, emphasize the imagination and technical advances being leveraged by researchers in the fight against malaria.

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REVIEW
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Novel Strategies for Malaria Vaccine Design

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The quest for a licensed effective vaccine against malaria remains a global priority. Even though classical vaccine design strategies have been successful for some viral and bacterial pathogens, little success has been achieved for *Plasmodium falciparum,* which causes the deadliest form of malaria due to its diversity and ability to evade host immune responses. Nevertheless, recent advances in vaccinology through high throughput discovery of immune correlates of protection, lymphocyte repertoire sequencing and structural design of immunogens, provide a comprehensive approach to identifying and designing a highly efficacious vaccine for malaria. In this review, we discuss novel vaccine approaches that can be employed in malaria vaccine design.

Keywords: *Plasmodium falciparum,* malaria, vaccine, immunoinformatics, structure-based, lymphocyte repertoire sequencing

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THE GLOBAL MALARIA SITUATION

Malaria caused by *Plasmodium* parasites remains a major infectious disease of public health importance. The disease is caused by five protozoan species, namely *Plasmodium falciparum*, *P. vivax, P. malariae, P. ovale,* and *P. knowlesi.* The deadliest of these is *P. falciparum* which is predominant in sub-Saharan Africa (SSA). In 2016, approximately $2.7 billion was invested globally in control and elimination programs (1). Meanwhile, it was estimated in 2016 that nearly half of the world's population was at risk of infection, with 91% of the estimated deaths being in Africa and 70% of the mortality occurring in children under 5 years (1). Notwithstanding, preventive control and intervention measures have helped decrease the burden between 2000 and 2015. For instance, the incidence of new malaria cases was down by 37% world wide and 42% for the WHO African region. In addition, the incidence of mortality over the same period decreased by about 60% globally and 66% for the African region (2). Yet, malaria imposes huge economic losses for people in the African Region and there is a need to upscale the available interventions and introduce new ones such as a licensed cost-effective vaccine (3).

CHALLENGES TO THE ERADICATION OF MALARIA

Malaria eradication faces many challenges including insecticide resistance, emerging anti-malarial drug resistance and the presence of asymptomatic and submicroscopic infections.

Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs), have been among the most effective tools for malaria control and elimination (4). So far, pyrethroids are the only

recommended class of insecticides for LLINs. However, more than 30 countries have reported resistance to pyrethroids, which has the potential to spread to new areas (5–9).

The rapid development of pyrethroid resistance suggests that alternative classes of insecticides need to be identified. As a result, WHO has cautioned against the use of pyrethroids (8), raising the need for alternative measures of control. The development of resistance to malaria drugs by *P. falciparum* remains a major threat to malaria elimination. The WHO-recommended first line treatment for uncomplicated malaria caused by *Plasmodium falciparum* is the artemisinin-based combination therapies (ACTs). Historically, *P. falciparum* has been able to develop resistance to almost all previous first-line antimalarial drugs (10, 11). The development of resistance to these drugs almost always begins from South-East Asia, where mutant parasites resistant to antimalarial drugs are more likely to survive due to lower levels of acquired immunity, poor adherence to administered drugs and higher parasite burdens (11–14). *P. falciparum* resistance to artemisinin-based drugs seems to have emerged sporadically (15), with mutations for resistance found within the kelch 13 propeller gene (15, 16). An inevitable fact is that artemisinin resistance may be imminent and other intervention avenues such as the development of highly effective vaccines need to be rapidly explored.

Also, the presence of asymptomatic and submicroscopic infections poses a major threat to malaria eradication and control. Continuous exposure to infectious mosquito bites leads to the development of anti-disease and anti-parasite immunity. The level of this immunity is determined by the transmission intensity and epidemiology of the disease (17, 18). It has been shown that the microscopic prevalence of malaria is almost half of that detected by nucleic acid amplification techniques and lower in low transmission areas (19, 20). The prevalence of submicroscopic infections has been found to be high in low transmission areas and common in children, probably as a result of a less robust immune response, leading to insufficient time for the development of protective immunity. In addition, asymptomatic infections may persist for several months and serve as a major threat to malaria eradication (21) as they sustain disease transmission (22–25).

CURRENT APPROACHES TO DEVELOPING A MALARIA VACCINE

Malaria Vaccines

The acquisition of partial immunity and the successful treatment of clinical symptoms of malaria in children with purified immunoglobulins from semi-immune adults (26) are positive indications of the feasibility of a vaccine against malaria. This is also supported by the induction of sterile immunity in both animal models and controlled human malaria infection (CHMI) through immunization with either live or attenuated sporozoites and merozoite-infected red cells (27–29). Attenuated sporozoites, even though they still maintain their natural hepatocyte invasion ability, do not fully mature in the liver and hence do not form merozoites that are responsible for the clinical symptoms of malaria (30).

Vaccine Targets

There are three stages to target for a potential malaria vaccine candidate. The first target of vaccine development is the pre-erythrocytic stage. This is the period where sporozoites travel through blood and infect hepatocytes to undergo schizogony, the vigorous multiplication stage that precedes the invasion of red blood cells (RBCs). The main purpose of developing a vaccine against this stage is to inhibit hepatocyte infections and hepatic parasite development, thus limiting RBC invasion (27, 30). The mechanisms of protection for this stage may involve antibody responses that prevent sporozoites from invading hepatocytes or cytotoxic T cells that destroy infected liver cells. So far, the licensed RTS,S, subunit vaccine remains the most advanced malaria vaccine to be developed. Other candidate vaccines include the whole-parasite vaccine candidates such as *Pf* sporozoite (PfSPZ), PfSPZ vaccination with chemoprophylaxis (PfSPZ-CVac) and the genetically attenuated parasite (PfSPZ-GAP).

The second target for malaria vaccine candidate design is the blood-stage of the parasite. The motivation for developing such vaccine candidates comes from evidence that people with repeated malaria infections in endemic areas develop some level of protective immunity, a state in which there is immune-controlled RBC invasion, resulting in fewer disease symptoms or asymptomatic infections (26, 31). Accordingly, vaccine candidates have been designed to elicit immune responses that will block/limit merozoite invasion of RBCs and stop the rapid replication of merozoites by targeting parasite surface proteins such merozoite surface proteins, apical membrane antigen 1 (AMA1), and the reticulocyte homolog (Rh) proteins (32–35). Other blood-stage vaccines target parasite antigens embedded in infected RBC membranes, such as *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) (36).

Despite being highly immunogenic and showing good promise as vaccine candidates, most of these antigens are also highly polymorphic and hence elicit antigen and parasite strain-specific responses (32, 33). Conversely, antigens such as the Rh proteins that show a high level of conservation (34, 35) tend to be less immunogenic (37).

The third malaria vaccine candidate target is the sexual parasite forms or gametocytes. Malaria transmission-blocking vaccines (TBVs) are designed to interrupt parasite transmission between humans and the mosquito vector through host immunological response to parasite targeted proteins such as Pfs230, Pfs45, Pfs48 (pre-fertilization antigens) and Pfs25, Pfs28 (post-fertilization antigens). Successful malaria transmission depends on the availability of infectious gametocytes in human

Abbreviations: ACTs, artemisinin-based combination therapies; PfEMP1, *P. falciparum* Erythrocyte Membrane Protein-1; MSP1, Merozoite Surface Protein; AMA1, Apical Membrane Antigen 1; Rh, Reticulocyte homolog (Rh); MHC, Major histocompatibility complex; HLA, Human leukocyte antigen; PfSPZ, *Pf* sporozoite; PfCVac, *Pf* chemoprophylaxis vaccination; PfGAP, *Pf* genetically attenuated parasite; TBV, transmission-blocking vaccines; BCR, B cell receptor; bnMAb, broadly neutralizing monoclonal antibody; TCR, T cell receptor; VIMT, Vaccine that interrupt malaria transmission; SSM-VIMT, Sexual, sporogenic or mosquito stage VIMT; PE-VIMT, pre-erythrocytic VIMT.

peripheral blood that can be taken up by mosquitoes during a blood meal. Studies have reported that the degree of infectivity of gametocytes to mosquitoes is based on the gametocyte density, drug stress, clonality of infection and immune defenses of the mosquito (38–42). However, according to Churcher et al. (38), even at very low densities, gametocytes remain infectious to mosquitoes. Also, it has been reported that in the human host, transmission can be stable at very low densities and is not directly proportional to the gametocyte density in peripheral blood (43, 44). Basically, a TBV exploits the fact that there is a functional immunological activity against the sexual stage parasite proteins which is able to reduce the infectivity of the parasite, thereby decreasing malaria transmission (45, 46). Vaccine candidates that seek to interrupt malaria transmission (VIMT) are of two main types: (1) sexual, sporogenic or mosquito stage VIMT (SSM-VIMT) candidates which are expected to interrupt human-to-mosquito transmission; and (2) the pre-erythrocytic VIMT (PE-VIMT) candidates, which are expected to interrupt mosquito-to-human transmission (47). Among the TBV candidates, only Pfs25 and Pfs230 have undergone clinical trials in human (48–51). Unfortunately, a major challenge with these candidate vaccines is the inability to elicit higher antibody titers. In regards, there are considerations to conjugate these candidate vaccines (50, 52).

Current Status of Malaria Vaccine Development

After decades of extensive research, the pre-erythrocytic stage vaccine, RTS,S has been licensed and is expected to undergo further testing in malaria endemic areas before possible approval for immunization. Currently, together with RTS,S, only 20 candidate vaccines are undergoing clinical trials (**Table 1**). For RTS,S, a recent evaluation on the safety and immunogenicity of the vaccine co-administered with the recommended expanded programme on immunization showed the vaccine to be safe and immunogenic with no related adverse events (58). The RTS,S/AS01 consists of a recombinant protein of the *P. falciparum* circumsporozoite protein (CSP) conjugated to a hepatitis B virus surface antigen. During clinical trials, the efficacy of the vaccine after 4 doses was observed to be 43.9% in children aged 5–17 months and 27.8% in children 6–12 weeks old (59). However, vaccine efficacy wanes with time and fails to meet the target set by the Malaria Vaccine Technology Roadmap (60). Consequently, other vaccination regimens such as the number of doses, time of immunization, and alternative approaches for vaccination are being evaluated (61).

Also, the R21, a virus-like particle vaccine which is a biosimilar of RTS,S consists of the CSP conjugated to a single hepatitis B surface antigen. The RTS,S-like vaccine has been shown to provide sterile protection in mice at very low doses. In addition, it was observed that most of the immune responses elicited against the candidate vaccine targeted the CSP in contrast to the hepatitis B surface antigen which is often targeted in the RTS,S vaccinated individuals (53). Importantly, this candidate vaccine is designed such that more epitopes of the CSP may be exposed to host immune system to enhance the efficacy of R21.

Furthermore, the whole sporozoite vaccine has been reported to provide significant protection against falciparum malaria. The whole organism candidate vaccine design approaches include the radiation-attenuated sporozoites (PfSPZ), whole PfSPZ with chemoprophylaxis (PfCVac) and the genetically modified sporozoites (PfGAP). Even though PfCVac showed complete protection to homologous *P. falciparum strain,* moderate protection has been observed with heterologous strains in non-exposed vaccines (62). Clinical trials with PfSPZ in endemic areas have been shown to be safe and well tolerated, however, inducing low level of immune responses compared to naïve individuals (63, 64). These may suggest that the breadth of immune responses to PfSPZ vaccines need to be increased by considering other vaccination regimens.

PROMISING APPROACHES TO MALARIA VACCINE DEVELOPMENT

Recent technological advances have greatly improved the prospects for designing an effective malaria vaccine through advances in high-throughput biology and computation. These alternative approaches may be focused on the parasite- or host immune system.

The Parasite-Focused Approach

The technologies involved in this approach center on the identification of immunogenic antigens from the pathogen by interrogating the parasite's genome, transcriptome or proteome. It may modify the structure of the antigenic component(s) identified with the aim of targeting various strains of the pathogen. The parasite-focused approach further tests the immunogenicity and safety of the candidate antigens to design novel and improved vaccines. This approach may involve the application of reverse vaccinology, structural vaccinology, and immunoinformatics.

Reverse Vaccinology

Reverse vaccinology, developed by Rappouli et al. is a technology first used in *Meningococcus* serogroup B bacteria to identify novel vaccine antigens (**Figure 1**). Here, the pathogen's genome is sequenced and analyzed to have access to the entire repertoire of proteins and enable comparison of conserved sequences shared among pathogens of the same species (65). Genomic data is analyzed using bioinformatics tools, taking into consideration all open reading frames. Also, with the use of computational tools, genomic sequences that are homologous to those of humans are eliminated from the vaccine candidates identified. The remaining genes are isolated and inserted into a suitable vector to obtain proteins for testing in animal models. Responses to the vaccine antigens are analyzed in immunized mice to validate their immunogenicity and efficacy levels. Importantly, molecular epidemiology studies are undertaken using various strains of the pathogen to ascertain whether the selected antigens are conserved or highly variable in a given population (66). This approach has been used to develop vaccines against serogroup B *Neisseria meningitidis* (67); and identify vaccine candidates for, *S. agalactia* and *S. pyogenes* (68, 69). This vaccine design approach

TABLE 1 | Current malaria vaccines in clinical trials.

Vaccine candidate	Clinical trial registration number	Clinical trial stage
PRE-ERYTHROCYTIC		
RTS,S/AS01	NCT01345240	Phase 3
R21/AS01B	NCT02600975	Phase 1
R21/ME-TRAP	NCT02905019 (53)	Phase 2
ChAd63/MVA ME-TRAP	NCT01635647 (54–56)	Phase 2
R21/Matrix-M1	NCT02572388/NCT02925403	Phase 1/2
PfSPZ Vaccine	NCT03510481	Phase 1
PfSPZ-CVac (PfSPZ Challenge + chloroquine or + chloroquine/pyrimethamine	NCT03083847	Phase 1
GAP 3KO (52-/36-/sap1-)	NCT02313376	Phase 1
BLOOD-STAGE		
pfAMA1-DiCo	NCT02014727 (57)	Phase 1
P27A	NCT01949909	Phase 2
PAMVAC	NCT02647489	Phase 1
PRIMVAC	NCT02658253	Phase 1
SEXUAL-STAGE		
ChAd63 Pfs25-IMX313/MVA Pfs25-IMX313	NCT02532049	Phase 1
Pfs25-EPA/Alhydrogel	NCT01867463, 51	Phase 1
Pfs230D1M-EPA/Alhydrogel and/or Pfs25-EPA/Alhydrogel	NCT02334462	Phase 1
Pfs25M-EPA/AS01 and/or Pfs230D1M-EPA/ASOI	NCT02942277	Phase 1
Pfs25 VLP-FhCMB	NCT02013687	Phase 1
Pfs25-Pfs25	NCT00977899	Phase 1
Pfs25 & Pvs/Monatide ISA 51	NCT00295581	Phase 1

Adapted from WHO. 9/28/2018. Malaria Vaccine Rainbow Tables. http://www.who.int/vaccine_research/links/Rainbow/en/index.html.

has greatly enhanced the discovery and characterization of several pathogen antigens.

Reverse vaccinology has been applied in malaria to identify parasite proteins either secreted or involved in signaling for consideration as possible vaccine candidates. The genomic sequence of *P. falciparum* has been available since 2002 (70). In addition, the sequence of other diverse *Plasmodium spp* including primate (71, 72) and rodent (73) parasites have been published. Comparative analysis of these sequences has shown similar homologs between species with possibly similar functions. For instance, the conserved Pf48/45 and PfHAP2 genes, both of which are transmission blocking vaccine candidates, functions were determined based on the role of their homologs in other *Plasmodium* spp. The functional analysis of the P48/45 and PHAP2 genes in *P. berghei* established their significant role in reducing the fertility of male gametes during fertilization, promoting these genes as vaccine candidates (74, 75), which are currently in the preclinical stage (76–78).

Despite the success of reverse vaccinology, it cannot be used to identify non-peptide antigens but can identify operons that code for synthesis of such molecules (79). For pathogens with complex genomes such as malaria parasites, no successful vaccine has as yet been developed via this approach. Further progress requires, among other things, improved predictive algorithms to identify the T and B cell epitopes as well as accurate quantitative assessments before inclusion in vaccines.

Structural Vaccinology

An improved understanding of the native structures of biological macromolecules such as proteins and how changes in their structure affect their functions can assist the identification of suitable epitopes (80, 81). Such epitopes can be designed into accessible forms for easy uptake by immune cells. These structural considerations make it possible to improve vaccine immunogenicity and safety and mitigate the effects of sequence variability within different strains of a pathogen (82). For instance, the bacterium *Meningococcus* is able to evade the host's immune system with the aid of a factor H binding protein (Hbp), which inactivates the host complement pathway by blocking factor H. Structural considerations allowed immunodominant epitopes of Hbp from various meningococcal strains to be identified and grafted into a single variant molecule to form a single antigen. This antigen was used in the MenB vaccine, responses against which are able to neutralize all the targeted strains (83).

Also, in an earlier study, short conserved α-helical coiled coil structural domains were identified from the asexual blood stage of the *P. falciparum* by examining the *Plasmodium* genome (84). Upon further screening, an unstructured peptide (P27A) that unfolds in native confirmation was selected. The peptide was the target of human antibodies which were able to restrict parasite replication (85). The vaccine candidate P27A has been considered immunogenic and safe with mild adverse events after Phase1 clinical trials (86).

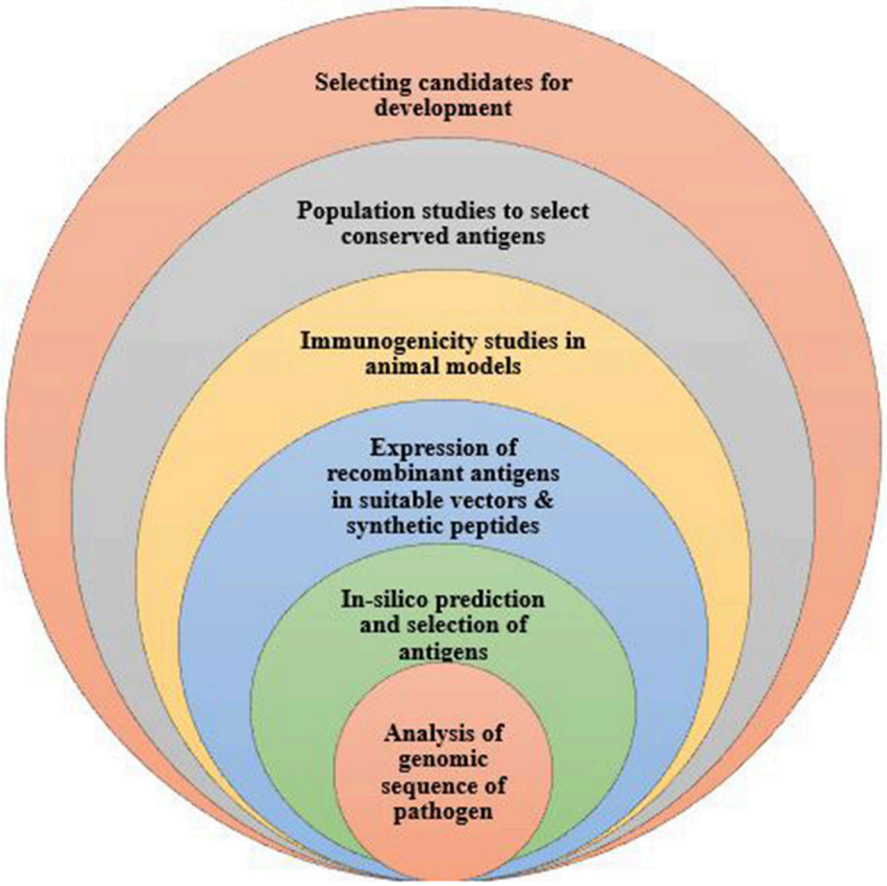

FIGURE 1 | The process of developing a vaccine using reverse vaccinology. Reverse vaccinology starts with obtaining the genomic sequence of the pathogen and using bioinformatics tools to identify all open reading frames to predict protein antigens. The predicted antigens are subsequently expressed in suitable vectors to produce the recombinant proteins which are tested to evaluate the immunogenicity in animal models. Expressed antigens that yield high immunogenicity are selected as vaccine candidates, further tested in population studies to determine and identify conserved antigens for further vaccine development.

For pathogens like *P. falciparum*, structural vaccinology may also help overcome antigenic variation. For instance, the application of structural vaccinology enabled the characterization of the less polymorphic DBL4ε domain of VAR2CSA to identify novel properties in the motif that affects the functional features of the antigen (87); identification and confirmation of the three-dimensional structure of the invasion ligand Cysteine-Rich Protective Antigen (CyRPA) (88). For example, the CyRPA was identified as a protective epitope providing an additive effect with the Reticulocyte binding-like Homologous protein 5 (*Pf*RH5) such that antibodies against *Pf*RH5 and CyRPA can inhibit parasite replication in host RBCs (88). Hopefully malaria vaccines incorporating these epitopes may elicit strong protective immune responses. Combining these protective antigens to create hybrid protein vaccines with enhanced efficacy may be a viable option for malaria.

A key challenge with this approach is the identification of suitable B and T cell epitopes for incorporation into vaccine candidates.

Immunoinformatics Based Approach to Vaccine Design

Immunoinformatics integrates both computational approaches and experimental immunology to develop machine learning algorithms that attempt to predict the immunogenicity of antigens. These approaches can be either pattern- or theory-based and may operate at either the amino acid sequence or the protein structure level. The pattern-based approaches conceive the prediction problem as one of finding sequence/structural patterns associated with immunogenicity. In contrast, the theory-based approaches attempt to model the basis for immunogenicity, for example, by using physical principles. Examples of algorithmic tools employed by pattern-based approaches include quantitative structure-activity relationship analysis, support vector machines, and artificial neural networks (89, 90). Theory-based approaches often employ Markovian and/or Bayesian models as well as models based on statistical mechanics (91).

Immunoinformatic approaches have already been applied to *P. falciparum* to predict possible cytotoxic T cell epitopes coupled

with HLA A/B molecules for malaria peptide vaccine design (92). For example, the PfEMP1 gene, a member of the var gene family has been associated with parasite evasion from host immune mechanisms due to its multiple variation and ability to bind to different host receptors (36). In a recent study, both *in-silico* and experimental approaches were used to identify antigenic epitopes from CIDR-1 and DBL-3γ conserved domains of PfEMP1. These epitopes were predicted to have good binding affinity to HLA molecules as well as the capability to induce IFN-γ, IL-4 secretion and T cell proliferation in exposed individuals (93).

Classically, HLA class I molecules optimally require peptides that are 8-10 amino acids long for presentation to CD8 T cells while HLA Class II molecules optimally require 12-25 amino acids long peptides for presentation to CD4 T cells. Of note high predictive accuracies have been achieved for bioinformatics methods for predicting peptide binding to HLA I molecules; whereas those for predicting binding to HLA II molecules require further improvement. An even greater challenge is prediction of peptide binding to B cell receptors for effective antibody responses. On-going work by us and other groups is aimed at addressing some of these challenges (91, 94). However, not all HLA binders are good epitopes for T cells and this poses a major challenge for approaches that predict HLA binders without considering the global picture of HLA-peptide-TCR interactions. Nonetheless, these computational approaches, which are quite cost-effective and are important down-selection tools in instances where there are too many peptides to evaluate experimentally, have the potential to aid in the development of effective vaccines against malaria.

Immune-Focused Approach

Due to the sophisticated immune-evasion mechanisms of *P. falciparum* that allow it to coexist with the host, vaccinologists require new paradigms in vaccine development. One such new paradigm that has been developed to target these pathogens is the immune-focused approach (**Figure 2**). In contrast to the parasite-focused approach, which centers on the pathogen of interest, this new approach seeks to harness the host immune system to more rapidly design effective vaccines. It focuses on studying the host immune system to discover protective immune signatures. It is expected that these protective signatures can be induced *de novo* in susceptible hosts to protect them against infection and/or disease. Compared to the pathogen-focused approach, the immune-focused approach has, in principle, a greater potential for success against pathogens like malaria parasites, which have highly variable genomes. In particular, it may be able to identify and design immune cells with broadly neutralizing antibodies (95) and enhanced cellular immune responses, which has proved difficult to accomplish by using conventional approaches. To provide context for the discussion of opportunities for vaccine development, we begin with a brief overview of human immunity to malaria.

Immunity to Malaria

In contrast to many pathogens against which highly potent, long-lived immunity is achieved, human immunity to malarial parasites is less potent and relatively short-lived (17). In malaria, acquired immunity to infection is rare; rather, what develops naturally, generally over a long period, is acquired immunity to disease (96). Such clinical immunity generally targets the disease-causing asexual blood stage of malarial parasites. It tends to be acquired faster in moderate-to-high transmission settings compared to low transmission areas, and with a higher number of clinical episodes (97–99). As with other infectious diseases, the development of clinical immunity to malaria is dependent on the adaptive arm of the immune system, and the principal mediators consist of specific subsets of B and T cells. Some progress has been made to elucidate the underlying mechanisms, although the key immune determinants remain unclear.

In addition, the ability to predict the beginning and end of transmission seasons have made it possible to study host responses to infection and some immune dynamics that occur before, during and after infection as well as, drug interventions (100) and how they may affect the immunity acquired (101, 102).

In natural infections, acquisition of immunity to sporozoite stage infections is limited, probably due to the low number of sporozoites that are inoculated as well as the limited time that sporozoites have extracellular, prior to hepatocyte invasion. In addition, it has been reported that *Plasmodium* sporozoites are able to modulate the cytokine environment by downregulating Th1 responses and antigen presentation to T cells (103). Recently, it was reported that continuous exposure to *P. falciparum* leads to the induction and expression of immunoregulatory cytokines such as IL-10 and affects the function of dendritic cells (104). These, coupled with frequent infection and immune activation, may profoundly impact on the tolerogenic environment leading to the escape of sporozoites from immune cells. Nevertheless, functional properties of antibodies to sporozoite-stage infections have been associated with natural protection from clinical disease. It has been reported that these antibodies kill sporozoites through complement fixation and inhibit hepatocyte invasion. However, the response to sporozoite antigens was age-dependent and acquired slowly compared to blood-stage antigens (105).

The blood-stage parasite is associated with the clinical symptoms of the disease as it causes an upregulation of pro-inflammatory cytokines, regulatory T cells and parasite sequestration in small blood vessels in host organs. Antibodies have been reported to play functional roles in preventing parasite invasion of red blood cells (106, 107). Antibodies to parasite antigens are associated with clinical immunity in endemic areas (108–110). The mechanisms of antibody activity may include blocking invasion of erythrocytes (111); opsonizing parasites to facilitate their clearance (110, 112) enhancing the killing of infected cells by monocytes (113); complement-mediated lysis of infected cells (114); and inhibiting adherence of infected erythrocytes to vascular endothelium (115). However, the generation of atypical memory B cells which have reduced effector functions has been observed under chronic conditions (116, 117).

T cells have also been shown to play protective roles during blood-stage infection. For instance, protection from the disease has been associated with $FOXP3^-$ Th1 cells which are self-regulatory and produce IFNγ, TNF and IL-10 (101, 118, 119). These cells are believed to prevent the production of pyrogenic

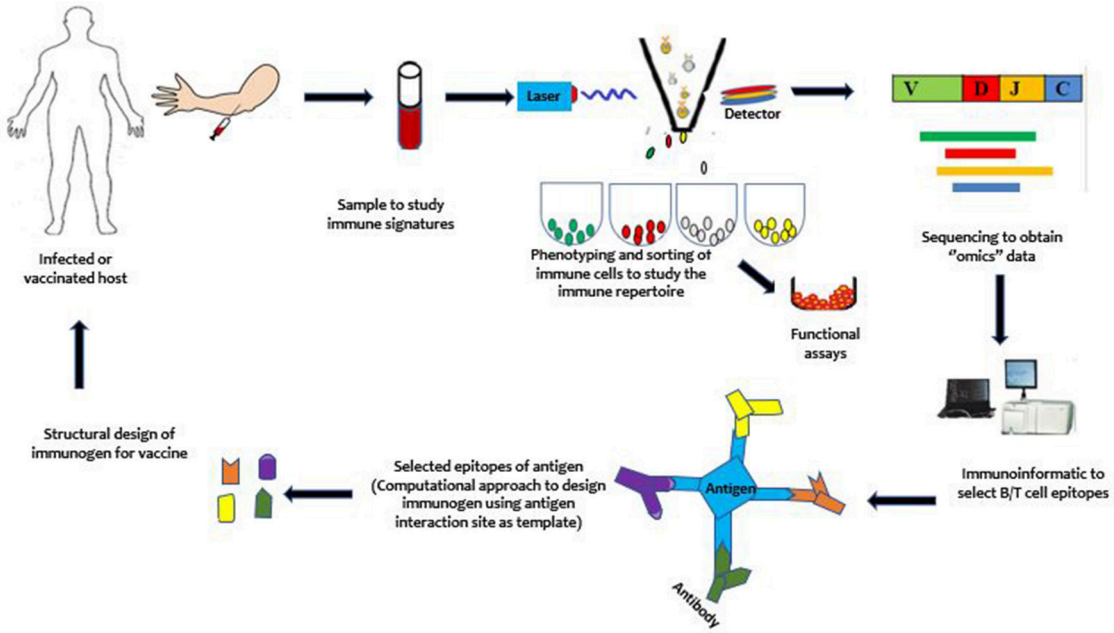

FIGURE 2 | An illustration of the immune-focused approach to vaccine development**.** Briefly, from top left, samples are obtained from an infected but protected or a vaccinated host. Immune cells are phenotyped and sorted using single cell sorting technologies such as the flow cytometry. The sorted cells may be sequenced directly or proliferated for functional studies before sequencing to study the immune repertoires (B/T cell receptor repertoire). With the use of computational approaches, the immunogen is designed and taken through further stages of development to yield a vaccine. After vaccination, mechanisms underlying vaccine efficacy and safety can be studied to further enhance the developed vaccine to achieve maximum protection against the targeted pathogen.

factors that may lead to the manifestation of clinical disease. However, these immune responses are not long-lasting and easily decay after infection has waned. T cell responses are hampered by the upregulation of negative immune regulatory receptors which may blunt or cause anergic responses (116, 120, 121). Our recent study found higher levels of T regulatory cells to be associated with higher blood levels of *P. falciparum* in children (118), suggesting less effective control of the parasite. Indeed, trying to understand these various aspects of the immune responses is a quite complex task (122).

Compared to natural infections, inducing sterile immunity in naïve individuals has been achieved through whole sporozoite immunization (29, 123) although similar outcomes have not been seen in individuals from malaria endemic areas (63, 64). Vaccination of volunteers with radiation-attenuated sporozoites has shown that both T cells and antibody responses play a significant role in protecting vaccinated cohorts against clinical challenge. It was observed that T cells from the periphery of these individuals, when stimulated with *P. falciparum* sporozoites *in vitro*, produced effector cytokines in a dose-dependent manner whereas antibody levels increased and prevented hepatocyte invasion (124–127).

The challenges of inducing immunity to malaria by natural or artificial means are compounded by the sophisticated immune-evasion strategies of the parasite. The parasite has a large genome consisting of about 5,300–5,500 possible antigenic targets (70). This extensive gene repertoire coupled with the parasite's high mutation rate allows for extensive variation of antigens that can be potential vaccine targets. Moreover, the epitopes targeted by the immune system exhibit a hierarchy of immunogenicity, with immunodominant epitopes that induce large amounts of antibodies, not all of which are neutralizing and may mask sub-dominant epitopes bound by neutralizing antibodies (73). In addition, the parasite switches off antigenic phenotypes, associated with the variant antigens resulting in functional diversity. Consequently, infections are mostly characterized by successive parasitemia waves caused by different parasite variants, making the development of long-lived immunity to the parasite very challenging (36, 128). Furthermore, key antigens such as CSP contain tandem repeats that have been implicated in immune evasion by suppressing antibody responses against adjacent antigens (129).

High Throughput Identification of Immune Correlates of Protection

The age and genetics of a person may modulate the immune responses elicited during infections and vaccinations (130, 131). Nonetheless, these responses that modulate infection may help to systematically define factors associated with protection from disease. Conventional approaches to understanding immune correlates of protection against *P. falciparum* includes, but is not limited to, ELISA, Elispot and Western blots. However, recent advances in high throughput assays have allowed in-depth analysis of immune correlates of protection to multiple *falciparum* antigens. Individuals in malaria-endemic areas

generate antibodies to different *P. falciparum* proteins which may be protective or serve as a serological marker for exposure.

High throughput assays that probe the genomic, proteomic and transcriptomic data of immune responses are useful means of determining correlates of protection in exposed and vaccine trial cohorts. Independent studies using library expression and protein microarray has characterized host immune reactivity to different *P. falciparum* antigens. Using these approaches, Doolan et al. (132) were able to identify stage-specific *P. falciparum* antigens associated with protection in naturally exposed individuals, vaccine protected and non-protected individuals using a protein microarray chip with 250 proteins. They observed distinctive antibody profiles in the various groups to these antigens. Also, in an independent study, involving a large cohort of children naturally exposed to malaria (≤10 years old) in Kenya, it was observed that responses to fewer proteins from the 39 *P. falciparum* antigens analyzed were significantly associated with protection, and these included AMA1 and MSP2. Also, antibodies to the top 10 proteins provided an additive effect whereas most antibody responses to the other antigens were markers of malaria exposure (133).

A similar study conducted in Mali probed sera from malaria-exposed children and adults against 1204 proteins. Among these proteins, 91 were associated with sexual stage-specific immunity with specific-IgG responses culminating during the transmission season. It was further observed that immunity to these sexual stage vaccine candidates (Pfs48/45 and Pfs230 but not Pfs25) can be boosted in natural infections (134). These studies showed evidence that the breadth and magnitude of the antibody response is a better correlate of immune protection. Furthermore, in analyzing PBMCs for non-humoral immune responses associated with protection using DNA microarrays, qRT-PCR and flow cytometry, it was observed that repeated exposure to malaria in children was associated with the upregulation of genes involved in immune regulation (such as IL-10 secretion from CD4+Foxp3-), phagocytosis and activation of adaptive immune system. In contrast, gene expression levels of chemokines and cytokines associated with fever and inflammation (such as IL-1β, TNF, CXCL2 and IL-8) were downregulated (101).

Interestingly, the application of next-generation sequencing techniques such as lymphocyte immune repertoire sequencing, including T cell receptor (TCR), membrane-bound B cell receptor (BCR) or secreted BCR can allow an in-depth analysis of host factors associated with pathogen recognition, identification and protection from disease. The TCR structure is heterodimeric with two protein subunits; an alpha and beta chain or gamma and delta chain with both a constant and variable region. Similarly, the BCR consists of two heavy and light chains which are joined together by disulphide bonds to form a Y shaped immunoglobulin together with a variable and a constant region. The lymphocyte receptors (TCR/BCR) have similar structures including a variable, diversity and joining regions that enable diversification in identifying different host pathogens. In the generation of receptor diversity, there is a recombination of a V, D, and J segment of a beta or heavy chain, and a V and J segment for the alpha or light chain. For the BCR, this process helps expose very potent neutralizing antibodies that may be public in protecting against clinical disease. The generation of the variable regions may help guarantee higher levels of somatic mutation at the antigen binding site which may be shared or unique to an individual(s).

Despite the documented importance of lymphocyte receptors for antigen recognition and, hence, for the initiation of adaptive immune responses, the specific TCRs/BCRs that determine immunity to particular pathogens remain poorly understood. To our knowledge, no previous study has comprehensively mapped these receptors and analyzed how their expression profiles may correlate with individual variations in immune protection against malaria. In addition, the application of machine learning algorithms such as random forests, support vector machines may allow the identification of patterns on immune correlates that may predict protection against disease (118, 135).

Moreover, these approaches generate huge amounts of data that can be computationally analyzed to generate new, experimentally testable hypotheses. These may yield novel insights into the mechanisms underlying vaccine safety and efficacy. Importantly, data from such studies will inform pathways to which vaccine strategies should focus.

The B Cell Response and Vaccine Design

Effective vaccines are supposed to elicit and provide long-term protection as well as require both B and T cells to produce effective antibodies to neutralize surface-expressed antigens. B cell lineage vaccine design is an immune-focused approach that combines human immunology, structural biology, and computational protein design to develop a vaccine. The aim is to identify in both naïve and memory B cell receptors, paratopes (antigen binding sites) that interact with immunogens of interest. For a vaccine to be designed through this approach, memory B cell clones from the same lineage (or clone) are first identified and isolated from patients that produce broadly neutralizing antibodies or protective antibodies. These clones are then sequenced to obtain the V(D)J and VJ gene pairs that make up the B cell receptors in order to identify the paratope. Computational approaches are used to design an immunogen that interacts with the identified paratope (95). For *P. falciparum,* neutralizing antibodies produced by activated B cells are required to prevent the infection of new RBCs. By isolating such protective B cells from malaria patients and sequencing and analyzing their antigen receptors, it might be possible to identify immunogens able to induce protective immunity in susceptible individuals.

In malaria, the identification of broadly neutralizing antibodies remained elusive partly due to the high polymorphic nature of *P. falciparum* antigens. In addition, malaria vaccine candidates tend to induce antibodies with weak neutralizing ability, low breadth and strain-specific. However, Tan et al. (136) have recently identified monoclonal antibodies that can recognize *P. falciparum*-infected RBCs (iRBCs) from different strains of parasites. These antibodies recognize and bind to iRBCs through the RIFIN proteins, a group of variant antigens that are extracellularly expressed on the surface of iRBCs and have been associated with immune evasion (128) to initialize opsonization.

Another remarkable example is the identification of the novel antigenic target NPDP (part of the sequence in the N-terminal

junction peptide) that is found between genes for the CSP and NANP and NVDP tandem repeats (137, 138). Independent studies in 2018 by Tan et al. (137) and Kisalu et al. (138) identified and isolated neutralizing antibodies from memory B cells and plasmablasts that could inhibit hepatocyte infection by PfSPZ. Through structural information, they were able to identify that these antibodies bind to conserved epitopes in the N-terminus of the CSP that is not found within the RTS,S vaccine.

Furthermore, mAbs that can inhibit parasite replication to about 97% have been isolated from CHMI donors immunized with RTS,S. Deciphering the structure and functionality of these antibodies have provided an informed overview on the structure of the CSP *in vivo*. Thus provides positive implications in the design of CSP immunogens against *P. falciparum* (139). However, there are still unsolved questions on the antibody responses to the PfCSP which have been described to be protective (139) and non-protective (129) as well as more structural information is needed to induce such potent neutralizing antibodies during vaccination Nonetheless, it is very interesting since they have implications in designing immunogens that can target specific immune responses and probably improve the efficacy of the RTS,S vaccine.

T Cell Response and Vaccine Design

Protective immunity to malaria liver-stage infection has been attributed to T cells in both human and rodent models. In studying immune responses to malaria such as cerebral malaria, murine models have provided significant understanding of various immunological properties that have impacted our understanding of the immune activity in humans.

For instance, Lau et al. (140) characterize MHC-restricted TCR that have potential in enhancing antigen presentation to T cells to enhance T cell immunity. They developed a novel CD8+ T cell receptor to *P. berghei* termed PbT-I from transgenic mouse with immune specificity for liver-stage and blood-stage infections. Isolated TCR genes from Vα8.3 and Vβ10 were isolated from a restricted hybridoma T cell line generated from *Plasmodium berghei* ANKA (PbA) blood-stage infection. Despite been developed for PbA, this transgenic MHC-I restricted T cell line was cross-reactive to *P. chabaudi* and *P. yoelli*. This implies that they may recognize conserved regions in rodent *Plasmodium spp*. Functional analysis revealed that the PbT-I cells produced effector cytokines (IFNγ, TNFα) and was positive for the degranulation marker (CD107a) showing their involvement in immune activity during the PbA infection. Using PbT-I CD8+ T cells, the peptides responsible for their activation were elucidated.

In a subsequent research, they identified and developed PbT-II CD4+ T cells from mouse transgenic line using the TCRα (Vα2.7, Jα12, Cα) and TCRβ gene (Vβ12, Dβ2, Jβ2.4) segments to blood-stage PbA infection (141). These cells were cross-reactive to rodent parasites (*P. berghei, yoelli and chabaudi*) and to *P. falciparum*. These MHC-II restricted PbT CD4+ T cells enhanced both humoral activity of B cells and cytotoxic activity of CD8+ T cells. In addition, the study confirmed that immunity to antigens in both blood stage and liver-stage development can restrict parasite replication in the hepatic stage and characterize the impact of blood stage antigen presentation to T cells that can enhance such T-cell immunity during infection. The uses of these target antigens may delineate protective immune responses and possibly circumvent pathologic outcomes. More importantly, further work should be focused on identifying and understanding such broadly reactive *Plasmodium*-specific T cells in host infections.

Structure-Based Immunogen Vaccine Design

The structure-based vaccine approach can be employed in both the parasite and immune-focused approach. However, in the immune-focused approach, the principle is based on understanding the structural properties of the immune cell providing the desired response. Here, the properties of the antigenic binding site on the immune cell is studied at the atomic level (80, 81). By understanding these properties, the approach seeks to design and develop immunogens to target the protective response or develop these immune cells for use as interventions.

Structural-based vaccine design has aided in unmasking immunodominant epitopes in the haemagglutinin-stem of the influenza virus (142), the fusion protein in the respiratory syncytial virus (143) and CD4 binding site in HIV-1 virus. For example, identifying conserved immunogenic epitopes in HIV has been quite challenging. However, elucidating the structure of broadly neutralizing antibodies (bNAbs) has been very useful. Using NAb, subdominant epitopes in the CD4 binding site by the gp120 viral protein were identified. Probing the structure of the antigenic binding site on CD4, the structural properties helped in the development of a recombinant protein (RSC3) with specificity to the NAb. The RSC3 was further used to identify and isolate B cells that expressed broadly neutralizing antibodies with increased breadth. VRC01 and 3BNC117, highly potent monoclonal bnMAb with reactivity to about 91% to HIV-1 isolates were developed (144). Phase I clinical trials of the VRC01 were reported as safe with no allergenicity (145, 146). It is currently being evaluated in a Phase IIB trials with a projected overall efficacy of 53 and 82% (147). These observations indicate that using structural properties, subdominant epitopes can be uncovered to design immunogens to target a specific immune response.

Currently, there are few examples of the successful use of these approaches in malaria vaccine design. For instance, using invasion-inhibitory monoclonal antibodies, the novel structure of PfRh5 in complex with basigin was characterized, together with novel protective epitopes found in the complex (148). Similarly, for *P. vivax* infections, bNAbs that confer strain-specific immune responses (149) were isolated. These bNAbs, enabled the characterization of protective epitopes in the duffy binding protein that can be included in the design of a potent *P. vivax* vaccine (150).

CONCLUSION

The development of a highly efficacious malaria vaccine faces many challenges, both technical and biological. Partly because

the parasite is equipped with a variety of evasion mechanisms allow it to co-exist with the host. With the recent advent of high throughput approaches such as lymphocyte repertoire sequencing and structural design of immunogens, the breadth of protection of previous and current vaccine candidates may be enhanced as well as the identification of new candidate vaccines. In addition, vaccinologist may be able to design vaccines that drive the immune system through unusual yet protective pathways. Likewise, the application of mathematical modeling and computational approaches to the data thus obtained will open new pathways toward designing highly effective vaccines against malaria and aid in achieving the targets set by the malaria vaccine technology roadmap for 2030.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Development of Whole Sporozoite Vaccines for *Plasmodium falciparum* Malaria

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Each year malaria kills hundreds of thousands of people and infects hundreds of millions of people despite current control measures. An effective malaria vaccine will likely be necessary to aid in malaria eradication. Vaccination using whole sporozoites provides an increased repertoire of immunogens compared to subunit vaccines across at least two life cycle stages of the parasite, the extracellular sporozoite, and intracellular liver stage. Three potential whole sporozoite vaccine approaches are under development and include genetically attenuated parasites, radiation attenuated sporozoites, and wild-type sporozoites administered in combination with chemoprophylaxis. Pre-clinical and clinical studies have demonstrated whole sporozoite vaccine immunogenicity, including humoral and cellular immunity and a range of vaccine efficacy that depends on the pre-exposure of vaccinated individuals. While whole sporozoite vaccines can provide protection against malaria in some cases, more recent studies in malaria-endemic regions demonstrate the need for improvements. Moreover, challenges remain in manufacturing large quantities of sporozoites for vaccine commercialization. A promising solution to the whole sporozoite manufacturing challenge is *in vitro* culturing methodology, which has been described for several *Plasmodium* species, including the major disease-causing human malaria parasite, *Plasmodium falciparum*. Here, we review whole sporozoite vaccine immunogenicity and *in vitro* culturing platforms for sporozoite production.

Keywords: *Plasmodium falciparum,* malaria, whole sporozoite vaccines, genetically attenuated parasite, radiation attenuated sporozoite, *in vitro* culturing

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INTRODUCTION

According to the 2016 World Malaria Report by the World Health Organization (WHO), nearly half of the world's population live in areas at risk of malaria transmission (1). The people most impacted by malaria are children under the age of five and pregnant women living in sub-Saharan Africa. In 2016, *Plasmodium falciparum* (Pf) caused the majority of the 445 thousand deaths and more than 200 million clinical cases, despite recent progress in malaria control efforts, which include vector control with insecticidal bed-nets and insecticide spray and chemoprevention with anti-malarial drugs (1). While these efforts have reduced morbidity and mortality caused by the disease, their impact has declined since the number of deaths and clinical cases have remained constant in the recent years highlighting the need for an effective vaccine. Vaccines represent the most effective form of control for certain infectious disease as demonstrated by the eradication of smallpox and near eradication of polio. However, vaccine development against

Plasmodium has proved more challenging than vaccine development against simpler bacteria or viruses. *Plasmodia* are complex eukaryotic parasites with large genomes (5,300 genes) and transform through multiple life cycle stages with stage-specific gene (antigen) expression (2). Thus, malaria vaccine development has been more challenging. For example, RTS, S/AS-01 (RTS, S) the most developed malaria vaccine candidate, which elicits antibodies against the major sporozoite (SPZ) surface protein, circumsporozoite protein (CSP), only provides partial efficacy (3, 4).

WHOLE SPOROZOITE MALARIA VACCINES IN DEVELOPMENT

Whole sporozoite vaccines (WSV) have demonstrated a range of vaccine efficacy that depends on prior exposure to malaria and ranges from ∼35 to 100% efficacy, in which malaria naïve volunteers are better protected. WSV in development include radiation attenuated sporozoites (RAS), genetically attenuated parasites (GAP), and WT sporozoites administered under drug cover (known as CPS–chemical prophylaxis with SPZ, ITV–infection treatment vaccination, or CVac–using Sanaria's injectable SPZ; hereafter referred to as CPS) (**Figure 1**). These live SPZ infect hepatocytes but do not lead to an established blood stage infection either by arresting in liver stage development (RAS and GAP) or by being eliminated during the initial stages of red blood cell (RBC) infection (CPS). WSV elicit immunity during two stages of the parasite life cycle, extracellular SPZ and intracellular liver stage for GAP and RAS and up to the initial stages of RBC infection for CPS. Thus, WSV can elicit immunity without causing clinical malaria symptoms. RAS delivered by bites of irradiated mosquitoes were first tested in humans in 1973 and protected humans from *Pf* malaria challenge (5). Since then, Hoffman and colleagues have made significant progress to produce vialed SPZ from aseptic mosquitoes for clinical testing of RAS (PfSPZ Vaccine) and CVac [Reviewed in (6)]. A recent PfSPZ study involving malaria-experienced individuals in Mali, in which 66% of the Malian vaccine group developed malaria infection, demonstrated reduced efficacy compared to trials involving malaria-naïve individuals (7). Thus, the PfSPZ vaccine failed to provide adequate protection in pre-exposed individuals. This study highlights the need for an improved dosing strategy, immunogenicity enhancement, and/or alternative vaccine approach for populations in malarious regions.

Alternative approaches include other WSV types described here and immunogenicity enhancement of WSV and subunit vaccines described in the following section (Whole Sporozoite Vaccine Immunogenicity). Current GAP in clinical development include early-arresting liver stage parasites that arrest at a similar developmental stage as RAS (<24 h) (8, 9). More recent *P. yoelii* pre-clinical GAP studies have engineered late-arresting liver stage GAP (2–3 days) (10–12). Whole parasites that arrest later in liver stage development (late-arresting GAP) or progress to the blood stage (CPS), provide a larger biomass and broader repertoire of immunogens, permitting lower SPZ doses for protection. For example, clinical CPS studies showed volunteers required 10- to 100-fold fewer parasites than RAS to achieve complete protection against malaria challenge, and pre-clinical late-arresting GAP studies showed that at least 10-fold fewer parasites could provide sterilizing protection and also protect against blood stage challenge (12, 13). Broad protection of late-arresting GAP was demonstrated by immunization with late-arresting *P. yoelii* GAP, which protected against cross-species challenge using *P. berghei* SPZ (11); however, heterologous challenge after CPS vaccination resulted in only modest protection after challenge (14). *Pf* GAP3KO safety has been demonstrated in a human clinical trial in which parasites arrested during liver stage development as all ten volunteers remained blood stage negative (8). However, in a previous safety trial using the first developed GAP, GAP2KO ($p36^-$ and $p52^-$) one in six volunteers showed patent blood stage infection (15). GAP2KO arrest later in liver stage development compared to GAP3KO (16), thus highlighting the challenge in developing a late-arresting GAP that is also completely liver-stage attenuated. Nevertheless, several groups are working on the development of late-arresting GAP due to their potential benefits.

WHOLE SPOROZOITE VACCINE IMMUNOGENICITY

Pre-clinical and clinical studies using GAP, RAS, and CPS have demonstrated safety of vaccination with WSV, immunogenicity, and vaccine efficacy [(17); Reviewed in (6, 8, 18)]. WSV immunogenicity data supports $CD8^+$ T cells as playing a critical role in protection. $CD4^+$ T cells, γδ T cells, and antibodies are also elicited after immunization, but their roles in the protective immune response are less clear. Complete reviews focused on WSV immunogenicity and potential clinical correlates of protection are discussed elsewhere (19, 20).

Cellular Immunity: $CD8^+$, and γδ T Cells

Numerous studies support roles for cellular immunity in WSV protection, including $CD8^+$, γδ, and $CD4^+$ T cells. The strongest immunological data supports roles for cytokine-producing $CD8^+$ T cells, liver resident $CD8^+$ T cells, and γδ T cells as possible WSV immune correlates during liver stage infection. For example, in rodents, depletion of interferon-gamma (IFN-γ) or $CD8^+$ T cells blocked RAS mediated sterile immunity (21). Subsequent studies were performed to analyze cellular responses and cytokine production after immunization by obtaining peripheral blood mononuclear cells (PBMC) and stimulating with *Pf* specific antigens, whole SPZ, or infected RBC. For *Pf*GAP2KO studies, interferon-γ (IFN-γ) producing $CD8^+$ T cell responses increased after immunization (15). For PfSPZ Vaccine immunization, levels of $CD8^+$ T cells that

Abbreviations: CHMI, controlled human malaria infection; CPS, chemoprophylaxis with *Pf* sporozoites; CSP, circumsporozoite protein; CVac, CPS using Sanaria's injectable sporozoites; GAP, genetically attenuated parasite; IFN-γ, interferon-γ; IL-2, interleuken-2; ITV, infection treatment vaccination; IVS, *in vitro* sporozoite; NHP, non-human primate; *Pf, Plasmodium falciparum*; PMBC, peripheral blood mononuclear cells; RAS, radiation attenuated sporozoite; RBC, red blood cell; SPZ, sporozoite; RPMI, Roswell Park Memorial Institute; RTS, S, RTS, S/AS-01; SPZ, sporozoite; TNF-α, transcription factor α; WHO, World Health Organization; WSV, whole sporozoite vaccine.

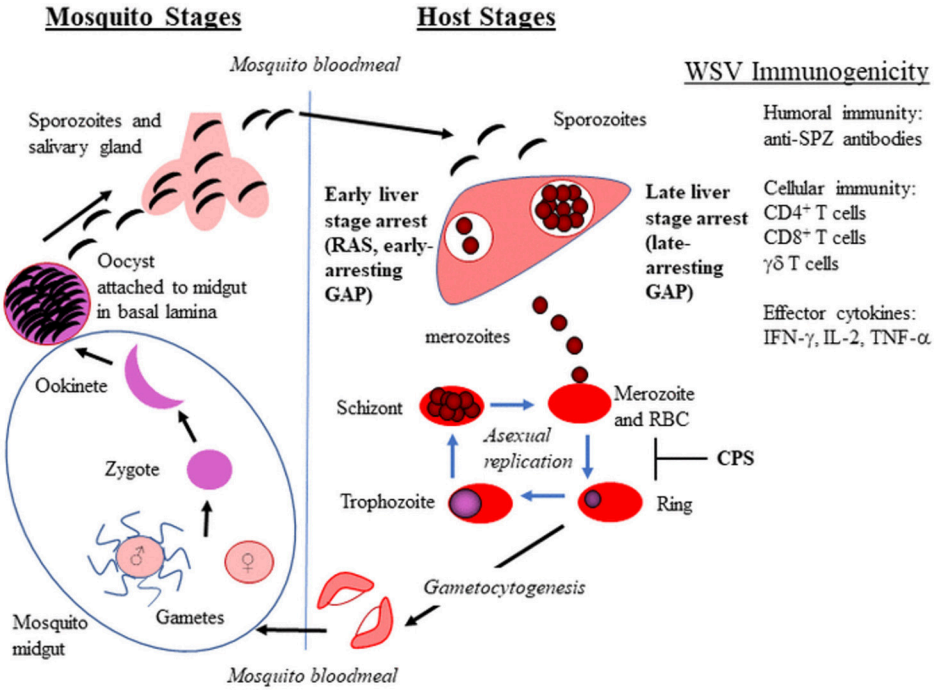

FIGURE 1 | *Plasmodium* life cycle in mosquitoes and vertebrate hosts. The whole sporozite vaccine (WSV) strategy for radiation attenuated sporozoites (RAS), genetically attenuated parasites (GAP), and chemoprophylaxis with WT sporozoites (CPS) is shown in bold. The WSV immunogenicity provided by WSV administration is also listed.

produced effector cytokine molecules [IFN-γ, interleuken-2 (IL-2), or transcription factor-alpha (TNF-α)] increased compared to pre-vaccination (22, 23). However, cytokine-producing T cell responses did not correlate with protection (22, 23).

Non-recirculating liver resident $CD8^+$ T cells represent an immunological marker that cannot be sampled in peripherally circulating cell populations (e.g., PBMC) from human clinical trials. Several studies support liver-resident and IFN-γ-producing $CD8^+$ T cells in providing protection after WSV administration. First, a PfSPZ immunization study was performed and involved different vaccine administration routes in mice, non-human primates (NHP), and humans (24). The greatest protection after CHMI was provided by IV vaccination compared to intradermal or intramuscular. Also, animal studies in mice and NHP identified IFN-γ-producing $CD8^+$ T cells in the liver, which correlated with protection by IV administration (24). Subsequent parabiosis experiments in mice confirmed the existence of liver-resident $CD8^+$ T cells (25), which provided RAS-induced malaria immunity (26). Further PfSPZ vaccination studies of NHP support liver resident IFN-γ-producing $CD8^+$ T cells in providing immunity as these cell levels were ∼100-fold higher in liver than in blood (27). Finally, several pre-clinical and clinical PfSPZ and CVac studies have also identified γδ T cells as possible correlates of protection that increase after subsequent vaccinations (22, 23, 27, 28).

While WSV have reduced immunogenicity and efficacy in some pre-exposed individuals (7, 29), vaccination strategies are underway to improve WSV immunogenicity. This work was motivated by numerous studies that support liver-resident $CD8^+$ T cells as primary mediators of long-term pre-erythrocytic immunity. Several groups are developing either "prime and target" or "prime and trap" approaches in which peripheral $CD8^+$ T cells are first primed and then targeting to the liver by high expression of *Plasmodium* subunit antigens or RAS infection (30, 31). These methods lead to higher levels of liver-resident $CD8^+$ T cells and improved RAS or subunit vaccine efficacy compared to RAS or subunit alone (30, 31). Further clinical studies will determine whether these pre-clinical results translate to protect diverse populations of humans, especially in malarious regions.

Additional WSV immunogenicity studies must be performed to identify strong immune correlates for the purpose of guiding clinical trials, including dose and regimen strategies. These studies may be focused on identifying antigen specificity of important T cell populations (γδ and liver resident $CD8^+$ T cells). For example, sequencing studies of T-cell receptors that bind malaria-specific antigens may identify strong correlates of protection. Moreover, since liver resident T-cells cannot be studied using PBMC from human clinical trials, future studies using NHP will prove valuable in identifying strong immune correlates of protection provided by WSV.

In vitro Sporozoites for *Pf* Malaria Vaccines

While WSV are the most effective vaccines against *Pf* malaria, major hurdles prevent the manufacturing of sufficient quantities

at a commercial scale. Current methods of vaccination with SPZ include delivery by mosquito bite or by injection after technically challenging and laborious mosquito dissections. Neither approach is practical for large-scale or cost-effective vaccination, which is required for commercialization. Culturing systems that produce infective SPZ independent of mosquitoes would enable scalable production of *Pf* SPZ for WSV.

Plasmodium Stage Development in Mosquitoes

Continuous culture of blood stage malaria parasites (asexual and sexual) has been established for decades (32–35). However, the *in vitro* culturing of mosquito stages has proven extremely challenging. To culture SPZ, one must understand *Pf* development within the mosquito and recapitulate this in culture. *Pf* develops through five sequential stages in mosquitoes: gamete, zygote, ookinete, oocyst, and SPZ (**Figure 1**). Prior to mosquito stages, gametocytes are ingested by mosquitoes during a blood meal. After ingestion, gametocytes within the mosquito midgut emerge and transform into gametes that fuse to form a zygote. The zygote undergoes DNA replication and maturation into a motile and invasive ookinete that traverses the mosquito peritrophic membrane and midgut epithelium, and finally embeds into the basal lamina on the midgut periphery. The interaction between the ookinete and the mosquito midgut basal lamina is thought to trigger the transformation into the early oocyst (36–38). The sessile oocyst is the site of SPZ development (up to several thousand SPZ per mature oocyst). Once released, SPZ travel to the salivary glands through the hemocoel where they can be inoculated into a host during a mosquito blood meal (39).

As *Plasmodium* develops through multiple life cycle stages, studies indicate that transitions are driven by coordinated changes in stage-specific gene expression controlled by the apicomplexan Apetala2 (AP2) family of transcription factors (TFs) (40, 41). The 26 AP2 TFs represent the only known family of *Plasmodia* TFs and contain at least one AP2 DNA-binding domain (42). TFs controlling stage transitions include AP2-O, AP2-O2, AP2-O3, and AP2-O4 for ookinetes and oocysts (41, 43, 44), AP2-Sp, AP2-Sp2, and AP2-Sp3 for mature oocysts and/or SPZ (41, 45), AP2-G, and AP2-G2 for gametocytes (41, 46, 47), and AP2-L for liver stages (48). These TFs co-regulate expression of genes with common *cis* promoter elements enabling control of the *Plasmodium* life cycle (41). Understanding stage transition through gene regulation could improve SPZ culturing systems. Other culturing system improvements could include applying knowledge of the parasite's environment while developing in mosquitoes.

Plasmodium in vitro Sporozoite Culturing Systems

Although several publications already exist that describe *in vitro* culturing of *Plasmodium* SPZ for the mouse (*P. berghei*, *P. yoelii*), avian (*P. gallinaceum*), and human *(Pf)* species (49–52), there is only a single publication outlining culturing methods for each species and SPZ characterization. Specific details on the different *Plasmodium* culturing systems are provided in **Table 1**. Culturing system factors that likely support production of SPZ include co-culturing with *Drosophila* support cells (L2/S2), an optimal medium (Roswell Park Memorial Institute [RPMI] or Schneider's), and culturing on a gel-like surrogate for the mosquito basal lamina. All culturing systems required *Drosophila* co-culturing or using conditioned media (spent media) from L2/S2 cells. *Drosophila* cells may provide nutrients for the developing oocyst and/or oocyst capsule, including soluble nutrients, growth/signaling factors and/or extracellular matrix molecules that are normally present in the mosquito midgut basal lamina or hemolymph. For culturing media, the avian and human culturing systems utilized RPMI, which is also used for *Pf* blood stage culture. Subsequent studies on culturing the murine-specific *Plasmodia* determined that Schneider's media optimally supported *Drosophila* cell growth (49). Finally, for the basal lamina, all culturing systems except the *P. yoelii* system found the necessity of Matrigel, which is a basal lamina-like gel derived from murine Engleberth-Holm-Swarm tumor cells and consists of laminin, collagen type IV, heparan sulfate proteoglycan, entactin, and growth factors. Laminin and collagen IV are known components of the mosquito basal lamina and are considered important for ookinete binding and oocyst transformation for mosquito and *in vitro* development (36–38, 56). In sum, there are shared characteristics of the *Plasmodium in vitro* sporozoite (IVS) culturing systems that reveal important factors supporting axenic SPZ development, including *Drosophila* cells, Schneider's media, and Matrigel or a similar basal lamina.

Characterization of Cultured Oocysts and Sporozoites

For the different *Plasmodium* culturing systems, oocyst, and SPZ development have been characterized to varying degrees (**Table 1**). The study that describes *Pf* culturing demonstrated that IVS morphology was similar to mosquito-derived SPZ and that IVS expressed the major SPZ surface protein, CSP (52). However, IVS functionality in terms of motility or hepatocyte traversal/invasion/infection was not analyzed. Similarly, for *P. gallinaceum*, only morphology and CSP expression were described (51). Separate studies on the rodent malaria parasites, *P. berghei* (49), and *P. yoelii* (50) analyzed morphology and sporozoite invasion of hepatocytes in mice. For *P. berghei*, IVS were produced with similar morphology and mouse infectivity compared to mosquito-derived SPZ, and blood stage infection was also observed (49). For *P. yoelii*, IVS infected primary hepatocytes in culture at a low rate (1/10,000) and hepatocytes in mice but infectivity was reduced compared to mosquito-derived SPZ. Furthermore, blood stage parasites were only seen in 1/15 infected mice (50).

While these studies demonstrate the feasibility of producing IVS that are capable of hepatocyte infection, many researchers have failed to replicate mosquito-stage culturing of *Pf* and other *Plasmodium* species due to the complexity of *Plasmodium* mosquito stage development and the extreme scientific and technical challenges of establishing culturing systems. We and

TABLE 1 | Characteristics of *in vitro* sporozoite culturing systems for *Plasmodium gallinaceum*, *falciparum*, *berghei* and *yoelli*.

***Plasmodium* species (reference)**	**Oocyst /SPZ culture conditions**	**Basal lamina requirement**	**Oocyst diameter**	**Ookinete to oocyst conversion** *in vitro* **(early or mature)** / *in vivo* **provided for comparison**	**SPZ days observed**	**SPZ characterization**
P. gallinaceum	*Drosophila* cells required	Matrigel	Day 3–7 mm for elongate	10–30% (mature)	Days 10 to 22 peaking on Day 16	Morphology with anti-CSP staining
(51)	In RPMI media		Days 5 to 7–up to 30 mm for spherical and up to 40 mm for elongated	1–21%* (53)		
P. falciparum	*Drosophila* cells required	Matrigel	Day 7–15 to 20 mm	N.D.	Days 12 to 16	Morphology with anti-CSP staining
(52)	In RPMI media		Days 10 to 12–25 to 40 mm	0.4–1.3%* (54)		
P. berghei	*Drosophila* cells required	Matrigel	Day 15–up to 40 mm	68% (mature)	Days 15 to 28	Morphology with Giemsa staining; Infectivity in hepatocytes of mice observed with subsequent blood stage transition and then mosquito infection
(49)	In Schneider's media			0–18%* (53)		
P. yoelii	*Drosophila* cells or conditioned media required	Matrigel not required	Day 3–4 mm	7.1% (early)	Days 6 to 30	Morphology with anti-CSP staining; Infectivity of primary mouse hepatocytes and hepatocytes of mice observed with subsequent blood stage transition
(50)	In Scheider's media		Days 6 to 7–10 mm	0–75%* (55)		

The features, including culture conditions, parasites observed, conversion frequency from ookinete to oocyst, and details of SPZ characterization are outlined and compared between culturing systems. For oocyst diameter, the observed oocysts were spherical unless otherwise noted. N.D, not described; RPMI, Roswell Park Memorial Institute.
**The range is due to differences in Anopheles mosquito species tested.*

others, including Sanaria, have made further improvements on developing *Pf* IVS culturing systems to ultimately produce SPZ for use in WSV development. For example, our *Pf* culturing system uses the unique nutrient, insect (silk-worm) hemolymph (56). Oocysts and maturing SPZ may receive soluble nutrients, growth factors, and/or signaling factors from the insect hemolymph as from the mosquito hemolymph. Successful *in vitro Pf* conversion frequencies compared to mosquito development have been achieved for life cycle stages up to the mature oocyst stage (56). Current developing approaches are focused on improving conversion to the mature oocyst and SPZ stages by using 3-dimensional culturing on non-Matrigel basal lamina-like substrates. Matrigel presents challenges for cGMP vaccine production as it is tumor-derived and may vary between batches.

Summary and Future Considerations

Future hurdles for the production of IVS to use in WSV include demonstrating equivalent functionality compared to mosquito-derived SPZ in terms of hepatocyte invasion/infection using cell lines and human-liver chimeric mouse models (57). While *P. berghei* IVS exhibit infectivity comparable to mosquito-derived SPZ, *P. yoelii* IVS have decreased infectivity, and hepatocyte infectivity was not assessed for *Pf* or *P. gallinaceum* IVS. IVS may resemble immature mosquito midgut SPZ more so than fully infectious mosquito salivary gland SPZ, which further develop in the mosquito hemocoel and salivary gland (58). Additional cues may be necessary for the IVS culturing systems to produce fully infectious, mature SPZ for use as a WSV. Moreover, GAP or RAS IVS will also need to satisfy safety requirements in terms of liver stage arrest and demonstrate absence of toxins, pathogens, or other contaminants. Thus, hurdles must be overcome in the purification of IVS from the 3D basal lamina and from *Drosophila* cells while maintaining IVS functionality. If these issues can be solved, culturing of SPZ would provide scalable production of WSV for further development as *Pf* malaria vaccines.

AUTHOR CONTRIBUTIONS

LSI, YZ, JD, AMG, AMV, and AKG developed the manuscript. LSI and AKG drafted the manuscript. All authors read and approved the final manuscript.

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Vaccination With Sporozoites: Models and Correlates of Protection

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Despite continuous efforts, the century-old goal of eradicating malaria still remains. Multiple control interventions need to be in place simultaneously to achieve this goal. In addition to effective control measures, drug therapies and insecticides, vaccines are critical to reduce mortality and morbidity. Hence, there are numerous studies investigating various malaria vaccine candidates. Most of the malaria vaccine candidates are subunit vaccines. However, they have shown limited efficacy in Phase II and III studies. To date, only whole parasite formulations have been shown to induce sterile immunity in human. In this article, we review and discuss the recent developments in vaccination with sporozoites and the mechanisms of protection involved.

Keywords: ***Plasmodium falciparum,*** **malaria, sporozoites, protection, vaccine, models, correlates**

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INTRODUCTION

Malaria is one of the deadliest diseases, causing a major public health problem with high mortality and morbidity. In 2017, the World Health Organization reported 219 million clinical cases and 435,000 deaths (1). The use of different control interventions such as insecticide-treated bed nets, combination drug therapies and early diagnostics has greatly reduced malaria mortality worldwide (2). However, with increasing drug resistance and insecticide resistance, these efforts are insufficient to eradicate malaria globally (3, 4). It has become increasingly clear that there is no control intervention that can singly eradicate malaria. Multiple control interventions need to be in place simultaneously and a malaria vaccine is integral to global malaria eradication (5).

PARASITE LIFE CYCLE

Plasmodium parasites have a complex life cycle, infecting two hosts, the human and the mosquito. In the human host, the *Plasmodium* life cycle consists of two stages, the liver stage and the blood stage. Infected female *Anopheles* mosquitoes inject sporozoites into the dermis of their mammalian host upon feeding. Sporozoites then enter the bloodstream and migrate to the liver, where the liver stage begins. The sporozoites multiply in hepatocytes, eventually forming merozoites in vesicles called merosomes. These vesicles rupture and release the merozoites into the bloodstream, commencing the blood stage by infecting red blood cells (RBCs). It is the continual cycling of malaria parasites within the RBCs, and the immune responses directed against this stage of the parasite, that causes most of the pathologies observed in malaria infections. The malaria parasites are then transmitted back to the mosquito following blood feeding by a female mosquito. The sexual forms of the blood stage parasites, gametocytes, develop into male and female gametes which fertilize each other, eventually forming oocysts in the mosquito's midgut wall. The oocysts then lyse to release sporozoites, which migrate to the mosquito's salivary glands. When the *Anopheles*

mosquito takes a blood meal on another human, the injected sporozoites migrate from the dermis to the liver, thereby beginning a new cycle of infection.

VACCINES AGAINST MALARIA

The development of vaccines for malaria has been met with many difficulties. Despite decades of research efforts, there is still no available vaccine for human use. This has led to the development of a wide range of approaches, in the search for an efficacious malaria vaccine. These approaches can be broadly divided into three main categories: (1) whole parasite-based vaccines, (2) subunit vaccines, and (3) viral, bacterial and parasite vectors as delivery vectors.

Whole Parasite-Based Vaccines

Whole parasite-based vaccines have had considerably more success than other vaccines. Whole parasite-based vaccines contain all parasitic antigens. This approach allows the development of different types of immune responses. Whole parasites used for the vaccines are obtained by dissecting sporozoites from mosquitoes or harvesting asexual blood stages from culture. There are many technical, logistical, and regulatory hurdles associated with large scale production and delivery of whole parasite vaccines in the field. However, recent sporozoite vaccine trials have shown considerable progress in overcoming these hurdles (6, 7).

History

The development of malaria vaccines began with whole parasite-based vaccines more than a 100 years ago when the Sergent brothers used heat-inactivated *P. relictum* sporozoites to immunize canaries and obtained partial protection (8). This was followed by the work of Russell and Mohan where both cellular and humoral responses against malaria were induced in immunized domestic fowls (9). In 1946, Jules Freund invented the Freund adjuvant and formulated the vaccine by combining the adjuvant with formalin-inactivated-blood infected with *P. lophurae*, an avian malaria parasite, or *P. knowlesi*, a monkey malaria parasite (10, 11). The formulations showed promising efficacy. However, the toxic side effects of the Freund adjuvant have prevented its use in humans. The first attempt in humans was done by Heidelberger et al., using formalin-inactivated *P. vivax*-infected blood to immunize volunteers, however no protection was induced (12). These initial studies, though suboptimal, have paved the way for future whole parasite-based vaccine development.

Vaccination With Sporozoites

Among the whole parasite-based vaccine candidates, there is considerable research on the pre-erythrocytic parasites. The pre-erythrocytic stage is an asymptomatic phase. Very few sporozoites are injected and subsequently developed in the hepatocytes during natural infection in human volunteers (13). The idea of inducing an immune response that can neutralize sporozoites in the skin and circulation and prevent the penetration of a low number of sporozoites into hepatocytes or destroy a low number of infected hepatocytes during the asymptomatic phase make pre-erythrocytic stage vaccines attractive. By preventing the pre-erythrocytic stage development, the vaccines would prevent blood stage infection, hence preventing pathology. In addition, the pre-erythrocytic stage vaccines have had more success than the other stages, which provides more support for their development.

Whole sporozoite-based vaccines developed thus far in human include (1) irradiated parasites (6, 14), (2) genetically-attenuated parasites (15, 16), and (3) drug-infection-treatment vaccination (17, 18).

Vaccination with irradiated sporozoites

Irradiated sporozoite vaccine is the most clinically developed whole parasite-based vaccine, and also the most clinically developed pre-erythrocytic vaccine (17). The first few studies that showed definitive protective immunity with irradiated sporozoites were done in chicks and subsequently in mice (18, 19). This was later demonstrated in humans, where vaccination with irradiated sporozoites, *via* infective mosquito bites, protected 92% of the volunteers from infection (20–22). However, >1,000 mosquito bites are required to introduce sufficient irradiated sporozoites to induce the high level of efficacy. This prevented the development of this approach for mass vaccination.

More recently, delivery of cyropreserved irradiated sporozoites into the host by direct venous inoculation *via* needle and syringe, has been tested in humans and showed promising efficacy data (6, 17, 23). While four doses only protected 33% of the individuals (6), five doses protected 100% of the individuals (6). More studies to perfect the vaccination regimes would allow direct venous inoculation *via* needle and syringe to replace mosquito bites as a delivery system. Another hurdle with irradiated sporozoite vaccines is the need for a high dose of irradiated parasites. Vaccine dosage, vaccination regimen, and route of administration have been investigated in malaria-naive adults (24). In the study, administration of higher doses may further enhance protection— four intravenous immunizations with a higher dose of 2.7×10^5 irradiated sporozoites was found to be the most optimal, where 55% of vaccinated subjects remained uninfected following controlled human malaria infections (CHMI) 21 weeks after immunization. The timing of the CHMI following vaccination has also been found to be important, with vaccine efficacy being higher when CHMI was performed 3 weeks after immunization, instead of 21 weeks. While vaccination with irradiated sporozoites led to sterile protection in 100% (6/6) of vaccinated malaria naïve volunteers (6), irradiated sporozoites vaccination in malaria-endemic Mali yielded a lower protection (14). There are fundamental differences between the two studies, such as the first study examines protection against homologous challenge and the latter study examines protection against heterologous challenge. Naturally transmitted parasites are often different from the vaccine strain. Twenty-four weeks after final immunization regimen, five doses of 2.7×10^5 irradiated sporozoites protected 7 of 10 against homologous CHMI, but only 1 of 10 against heterologous CHMI (25), showing that

the vaccine efficacy against heterologous infection is markedly reduced. In addition, the findings also suggest that pre-exposure to the malaria parasites may prevent the establishment of a protective immune response since blood stage infection is able to induce immune suppression (26). This also further highlights the need for optimization of the required dose and regime in the target population to achieve robust and sterile protection.

The irradiation of parasites is a delicate process that requires the sporozoites to retain a certain degree of viability. Similar to heat-inactivated and frozen-thawed sporozoites, over-irradiated sporozoites do not induce protection (27, 28). Irradiating the sporozoites leads to DNA damage in the sporozoites with no or limited reduction on hepatocyte infectivity (29, 30). Although irradiation results in an inhibition of parasite DNA replication, ultrastructure modification and alteration in gene expression (31, 32), eventually leading to developmental arrest of the liver stage within hepatocytes (33, 34), it still allows for parasite antigen presentation and priming of immune responses.

Vaccination with genetically-attenuated sporozoites

Non-irradiated sporozoites have been postulated to be more efficacious as whole parasite-based vaccines because, as compared to irradiated sporozoites, they are able to progress to a later stage of pre-erythrocytic development, (35). By doing so, the host immune system is exposed to a wide repertoire of malarial antigens and thus able to target more of the pre-erythrocytic stage. To this end, research efforts have focused on developing ways, other than irradiation, to attenuate the growth of the parasite. Recent advances in *Plasmodium* research such as genetic manipulation have brought forward a new approach to attenuate parasites. Genetically-attenuated parasites are modified by deleting key essential genes that result in developmental arrest of the liver stage after hepatocyte infection, but do not affect parasite viability, mosquito infectivity, and sporozoite production in animal models (36). Inactivation of UIS3, UIS4, or P36p prevented the attenuated parasites from developing beyond the early pre-erythrocytic stage in mice (37–39). Vaccination of these live genetically-attenuated parasites offered sterile protection against a challenge with a wild type isolate. While promising, one of the major concerns for the development of genetically-attenuated parasites as vaccines is the possible occurrence of breakthrough infections. The first clinical trial using live genetically-attenuated parasites that lack the two genes, p52 and p36, led to breakthrough infections (15). Breakthrough infections in mice have also been observed for UIS4- and P36p-deficient parasites (37, 39). However, great progress has been made recently. Live genetically-attenuated parasites lacking three genes (p52–/p36–/sap1–; "PfGAP3KO") arrest early in liver-stage development, and were safe with no observed breakthrough infection following administration into human subjects by infective mosquito bites (16). Compared to irradiated sporozoite vaccine and early liver stage-arresting genetically-attenuated parasites, second generation genetically-attenuated parasites that arrest late liver stages have shown to demonstrate superior anti-malarial immunity following vaccination in mice by having a greater antigen repertoire (40, 41). These candidate vaccines could also have greater efficacy in humans, but this remains to be demonstrated.

Drug-infection-treatment vaccination

Drug-infection-treatment vaccination is the last approach. It involves vaccination with live wild-type parasites under drug prophylaxis, where the drug targets and eliminates the blood stage parasites. This approach allows full liver development and a limited initial blood stage development, thereby focusing immunity toward the liver stages. Pioneer mouse studies have, indeed, shown greater efficacy when the mice were vaccinated with live *P. berghei* or *P. yoelii* sporozoites under drug prophylaxis than when the mice were vaccinated with irradiated sporozoites—fewer inoculations and less sporozoites were required to induce sterile protection (42–44). Vaccination with live sporozoites under chloroquine prophylaxis is the most investigated vaccine formulation under this approach, and it has shown very promising efficacy data. The first study in humans examined the efficacy of the vaccine where the live sporozoites were introduced into the volunteers *via* infective mosquito bites (45). The study utilized CHMI and demonstrated sterile protection, where 100% of the volunteers were protected from infection following a wild-type sporozoite challenge. A subsequent CHMI study investigated the efficacy of the vaccine where the live sporozoites were intravenously inoculated *via* a needle and syringe. In this study, a dose-dependent protection was observed, where only three doses of 5.12×10^4 sporozoites were sufficient to protect all volunteers from the challenge (46). In addition to chloroquine, vaccination with live wild-type parasites under prophylaxis of other antimalarials has also been investigated. Primaquine (47), mefloquine (48) and artemisinin derivatives such as artesunate (49) have been used in place of chloroquine and vaccination with live wild-type parasites under prophylaxis of these antimalarials has demonstrated protective immunity against a homologous sporozoite challenge in mice and in humans.

However, there are various challenges with this approach such as the technical and logistical issues associated with generating sporozoites at large scale and field deployment. There are also concerns that the sporozoite injections might not be properly followed with antimalarial treatment, which could lead to sickness. In addition, while sterile immunity can be achieved against a homologous sporozoite challenge, protection was suboptimal when immunized volunteers were challenged with a heterologous strain (50, 51), suggesting that the protective immune mechanisms target polymorphic antigens. Nevertheless, the current published findings have demonstrated very encouraging efficacy data and suggest that vaccination with live sporozoites under chloroquine prophylaxis, following vaccination regime optimization, could potentially be the most efficacious sporozoite vaccine until date.

Subunit Vaccines

Subunit vaccines allow rational design of the vaccine to induce the desired immune effectors against the parasite. In addition, subunit vaccines are safe and generally easy to produce at large scale and to administer in the field. Hence, efficacious subunit

vaccines that offer long term protection are the preferred vaccines of choice.

Peptides and Recombinant Proteins

Subunit vaccines have been developed either as peptides, multi-peptide constructs or recombinant proteins. They can be based on a single parasite antigen or a combination of multiple parasite antigens, and often in a formulation that includes adjuvants. Most constructs have been designed for the circumsporozoite protein (CSP), a major sporozoite surface protein (52), as it is the first cloned malaria antigen (53, 54). Both peptides and multi-peptide constructs containing either the B epitope alone or both B and T epitopes induced protection in mouse models (55–58). However, when tested in humans, the peptide constructs did not induce significant protection (59). The main reason for the failure in humans is that the immune response to these constructs was genetically restricted by major histocompatibility gene (60) and thus could not induce an efficient immune response in most volunteers.

To ameliorate immunogenicity and protective efficacy, peptides and proteins have been designed to contain T cell epitopes from the parasites or an unrelated proteins recognized by many MHC genes (61), coupled to diverse chemical backbones, or fused with other proteins to create particle vaccines to be used with or without various adjuvants (62–67). All these new constructs demonstrated a high efficacy in mouse models but have yet to be validated in humans.

Of all the subunit vaccines, RTS,S, a CSP-based subunit vaccine, is the current most clinically-advanced malaria vaccine, being the only malaria vaccine to have progressed to the pivotal Phase III clinical trials (68). Enormous resources have been spent on rationally improving RTS,S efficacy, which include developing novel adjuvant systems. Multiple studies have demonstrated a need for RTS,S be formulated with adjuvants such as monophosphoryl lipid A and QS21, to achieve immunogenicity (69–71). This was followed up by a series of clinical trials, where significant progress has been made to improve immunogenicity and efficacy (72, 73). The RTS,S vaccine has been designed to inhibit the liver stage and prevent blood stage infection. RTS,S is, ultimately, formulated with a chimeric molecule based on CSP, fused to the S antigen of the hepatitis B virus, together with a potent adjuvant, AS01. The first Phase IIb trials performed in adult volunteers in the USA showed ∼50% protection against clinical malaria (69, 70, 74).

When tested in the endemic regions, RTS,S's efficacy against infection was less impressive and was of short-duration (<3 months) (75, 76). What is encouraging is that RTS,S/AS01 vaccination has been found to induce a significant reduction (∼60%) in the incidence of clinical infections in children in the same study (76). This finding encouraged GlaxoSmithKline and the Malaria Vaccine initiative, with financial support from the Bill and Melinda Gates Foundation, to further develop this vaccine for infants and young children in Africa. However, RTS,S efficacy against clinical malaria was later found to be suboptimal in malaria-endemic populations, with a vaccine efficacy against clinical infection of 36.3% in young children and 25.9% in infants (77–81). One possible explanation is that the CSP used in the vaccine contains several T cell epitopes, which are all highly polymorphic in parasite population in the field. Neafsey et al. elegantly demonstrated that the overall vaccine efficacy was very low in field settings with minimal matching of the CSP alleles in the field with the CSP allele in RTS,S (80).

Another explanation for the limited efficacy of the CSP-based vaccines is that it may not be the best antigen to induce protection. It is likely that other antigens may be better vaccine candidates, alone or in combination with the CSP. This hypothesis was supported by various studies, which showed that the sterile protection against a sporozoite challenge obtained in mice immunized with irradiated sporozoites was independent of the immune response against the CSP (82–85).

With the limited success of subunit vaccines developed thus far, there have been many efforts to identify new pre-erythrocytic (liver) targets for vaccine development using various approaches (86–90). These new antigens have shown encouraging efficacy data in animal models either alone (91, 92) or in combinations (93, 94), however efficacy in humans has yet to be demonstrated (95).

DNA Vaccines

DNA has been identified as a vaccine delivery system in the 1990s (96). This approach was quickly taken up, and DNA vaccines against CSP were developed and tested in human (97–99). Although the CSP-based constructs induced high levels of protection in mice (100), they had poor immunogenicity in humans (101). DNA constructs encoding multiple genes (102, 103) or epitopes were also developed (104). However, none of these constructs induced high level of protection against a sporozoite challenge (104, 105). To enhance the immune responses, malaria DNA vaccines were developed in combination with DNA constructs encoding for cytokines such as GM-CSF. Although some of these constructs had increased immunogenicity and efficacy in murine models (106), they did not induce protection against sporozoite challenge in human (105).

Viral, Bacterial, and Parasite Vectors as Delivery Vectors

Viral, bacterial and parasite vectors have been developed as delivery vectors for malaria vaccines. As these vectors are based on whole organisms, they usually do not need to be adjuvanted to stimulate the innate immune system which is necessary for the development of an optimal adaptive immune response (107). However, in some studies, various adjuvants have also been used to increase vector constructs immunogenicity (108).

The use of viral vectors as delivery vectors for malaria vaccines is the most common (109). Recombinant influenza viruses (110), pox viruses such as vaccinia virus (110, 111), Sindbis virus (112), yellow fever virus (113, 114), adenovirus (115), human cytomegalovirus (116) as delivery vectors have shown promising efficacy in animal models. Bacterial vectors such as *Salmonella* (117, 118), Bacille-Calmette Guerin (BCG) (119), *Shigella flexneri* 2A strain (120) as delivery vectors have also shown good immunogenicity and efficacy against sporozoite challenge in mice. Currently, only *Salmonella* vectors as delivery

vectors have been examined in humans, showing good safety profile and immunogenicity (121). Parasites such as *Leishmania* (122) and *Toxoplasma* (123, 124) as delivery vectors were also able to induce partial protection in mice. However, it remains to be seen if these vectors can induce protection in human.

Prime-Boost Combinations

To enhance humoral and T cell responses, various prime-boost strategies have been developed using combinations of different vaccine approaches. Vaccination with subunits or DNA constructs, followed by viral vectors or combination of viral vectors encoding one or multiple malaria antigens has been examined and efficacy has been demonstrated in mouse models (125–129). However, of all these combinations, only a few have shown significant efficacy in humans (104, 109, 130–133). Recent strategies, prime-and-target (134) and prime-and-trap (135), have shown that the best combination, that can induce high level of protection in mice, depends on the capacity to induce and maintain tissue-resident memory cells in the liver.

MODEL SYSTEMS

The use of experimental models has an important role in the development of vaccines. It is essential for the first assessments of safety, immunogenicity and potential protective efficacy of vaccine candidates. The early studies of malaria candidate vaccines utilized avian models, despite being a poor alternative to study pathogens with mammalian hosts. The eventual establishment of other malaria models has brought new insights and greatly facilitated malaria vaccine research. Current models used include: (1) mouse, (2) non-human primates (NHP), (3) humanized mice, and (4) human volunteers (CHMI).

Mouse Models

Until date, the traditional mouse model still remains the most commonly used model as it is less costly and more easily available. *P. berghei* and *P. yoelii* are two of the more commonly used rodent malaria species for *in vivo* and *in vitro* studies. It is often the starting ground for *in vivo* studies examining the development of pre-erythrocytic stage and whole sporozoite vaccine candidates. Human parasites such as *P. falciparum* and *P. vivax*, which contribute to the majority of the malaria global disease burden, display highly restricted host-cell tropism—they cannot establish an infection and develop the pre-erythrocytic stages effectively *in vitro* easily. A reproducible model of full development of the pre-erythrocytic stage has only been described in primary human hepatocytes (136). The mouse model allows the examination of the parasite infection in the liver *in vivo*, and the effectiveness of the whole sporozoite vaccine candidates to protect the host from infection. While vaccine efficacy in the mice does not necessarily predict vaccine efficacy in humans, there have not been any examples where an absence of vaccine efficacy in mice was contradicted by vaccine efficacy in humans. Quite a number of whole sporozoite vaccine candidates that are first identified to be protective in mouse models (19, 42, 44) have went on to be validated in humans (22, 45). The first study demonstrating that vaccination with a whole sporozoite vaccine candidate, irradiated sporozoites, can induce sterile protection from infective sporozoite challenge was in a *P. berghei* mouse model (19). This has been established as the gold standard as human volunteers vaccinated with irradiated *P. falciparum* sporozoites were found to develop protective immunity (22). Similarly, vaccination with live sporozoites under drug prophylaxis was also first identified to have promising efficacy in the *P. berghei* and *P. yoelii* mouse models (42, 44), before demonstrating sterile protection in all vaccinated human volunteers (45). A new approach to chemically attenuate sporozoites has been identified using the *P. berghei* model. This was performed by treating sporozoites with centanamycin, a DNA alkylating agent (137–139). This may also offer protection in humans, however further studies to validate its efficacy are still pending.

With the development of transgenic rodent malaria parasites, knock-in (KI) parasites expressing *P. falciparum* (140) or *P. vivax* (141) genes have been generated. These KI parasites allow the examination of the efficacy of immunogens (142–144) or antibodies against human malaria pre-erythrocytic antigens *in vivo* (145, 146).

While the mouse models have greatly contributed to the development of malaria vaccines, there are limitations. It is still largely unknown how relevant the mouse models are for the human parasite. The ability to interpret and draw conclusion from the mouse and translate it to the human remains unclear. There are major fundamental differences, both at genetic and proteomic levels, between the rodent and human malaria species, with the rodent parasite genomes missing orthologs for more than 730 *P. falciparum* genes (147, 148). In addition, there are differences in the both the liver and blood stages of infection. While the mouse parasites, *P. berghei* and *P. yoelii*, emerge from the liver after 2–3 days of infection, the human parasites, *P. falciparum* and *P. vivax*, require 7–10 days of pre-erythrocytic stage development (149). The formation of dormant pre-erythrocytic stages in *P. vivax* infections, which is a hallmark of *P. vivax* infections (150), is also not present in *P. berghei* and *P. yoelii* infections in mice, although liver forms of *P. yoelii* have been observed in the liver of their natural host, *Thamnomys gazellae*, at least 8 months post-sporozoite infection (151). Lastly, most murine studies are performed with genetically homogenous inbred mice with a limited MHC gene repertoire, which do not mimic the large genetic diversity of the human population. Laboratory mice are maintained in clean specific-pathogen—free facilities, hence the absence of the effect of environment (e.g., microbiome) on the mouse immune system may bias infection and vaccine studies (152). Taken together, while it is a powerful experiment tool, the traditional mouse model is not an ideal model, especially for studying pre-erythrocytic malaria vaccines.

Non-human Primates

Due to the limitations of the traditional mouse models, there have been substantial efforts to develop alternative animal models that are able to generate adequate parallels in an *in vivo* approach of the human immune system. Historically, non-human primates (NHP) have been used as the alternative model. Compared to the mouse, NHP share a lot more similarities with the human.

Simian *Plasmodium* species can infect various NHP species. In particular, human parasites can be adapted to NHP and some NHP can even support direct infection with *P. falciparum* and *P. vivax* (153). The *Aotus* monkeys have served as a valuable model. They can be infected by *P. falciparum* and *P. vivax* (154, 155). The NHP models are particularly important for assessments of *P. vivax* pre-erythrocytic stage vaccines (156), due to the formation of dormant pre-erythrocytic stages in *P. vivax* infections, which cannot be recapitulated in the mouse models. Simian malaria parasites, such as *P knowlesi* or *P. cynomolgi* in macaques, have also been used for immune and vaccine studies (157, 158). Vaccination with live sporozoites under chloroquine prophylaxis has shown promising efficacy data in Toque monkeys immunized with *P. cynomolgi* (159). However, the lack of availability, the high costs to maintain a colony and the restriction of utilization due to ethical issues limit the use of NHP, especially where large numbers are required.

Humanized Mouse

In the more recent years, the development of a humanized mouse as an animal model (160, 161) has greatly facilitated the study of human malaria research. These models mainly arise from the xenotransplantation of human hepatopoietic cells and/or tissues, allowing the long-term establishment of components of human immunity in permissive immunodeficient mice.

Using a human liver chimeric SCID/Alb-uPA mouse, studies on the pre-erythrocytic stages can be performed (162, 163). While it has been shown to be a viable model to study *P. falciparum* pre-erythrocytic stage development, the study of human malaria in this model is limited to the pre-erythrocytic stage as *P. falciparum* cannot transit from the pre-erythrocytic stages to the blood stages in this model (164). Other drawbacks include infertility of the mice due to the SCID/Alb-uPA immunodeficient background (165), and hepatotoxicity and high neonatal mortality due to the uPA transgene expression (166). These have made the generation of large number of the mice extremely costly and difficult.

Due to these drawbacks, an alternative, a human liver chimeric $FAH^{-/-}Rag2^{-/-}IL2R\gamma^{null}$ (FRG) mouse, has been developed. These mice can be bred relatively easily and do not suffer from hepatotoxicity. In addition, this model has been shown to support robust pre-erythrocytic stage infection and development (149). When human RBCs were transplanted into these mice, the new model supported the transition from a pre-erythrocytic infection to a blood stage infection (167). The NOD mice deficient for the IL2Rγ gene and transgenic for the thymidine kinase gene (TK-NOG) is another model that has also been developed. These mice do not suffer from liver failure. A transient injection of the drug gancyclovir induces a controlled ablation of the mouse hepatocytes. Treated mice are easily repopulated with human hepatocytes (168). These mice can also be doubly engrafted with human red blood cells and this allows the full development of *P. falciparum* in the liver and the transition to the blood stage. Interestingly, this model also supported the liver stage development of another human parasite, *P. ovale* (169).These findings are extremely encouraging as this raises the possibility of using these models to study a liver stage infection and also a combined liver and blood stage infection. While the use of the humanized mice offers many new possibilities to study human malaria biology in a non-human model *in vivo*, it is worth noting that these mice are immuno-compromised, which makes them unsuitable for vaccine immunogenicity and efficacy studies. Nonetheless, they have shown to be useful in passive transfer experiments to test antibody efficacy (170–172).

Studies on the development of humanized mice with a fully reconstituted immune system are underway (173). In fact, humanized mice that possess the human immune system (HIS) have been established for malaria research, using recombinant adeno-associated virus (AAV)-based gene transfer technologies (174). With functional human CD4 T cells and B cells (HIS-CD4/B mice), these HIS mice were able to produce a significant level of human IgG against *P. falciparum* CSP upon immunization (175). The HIS-CD4/B mice were also protected against infection from an *in vivo* challenge with transgenic *P. berghei* sporozoites expressing the PfCSP protein following immunization. While these models are essential pre-clinical models to understand immune responses against human malaria, it is worth noting that these HIS mice still retain mouse myeloid compartments that are likely to influence antigen presentation and immune cell residency, and *in vivo* vaccine efficacy can only be examined using transgenic rodent malaria parasites expressing selected *P. falciparum* proteins. New iterations of humanized mice that possess the humanization of the liver, bone marrow, lymphoid compartments, and human erythrocytes would be the ideal mouse model and would greatly help to understand human malaria parasite infection and immunology. It would be an essential tool in providing a more accurate initial assessment of the safety profile and vaccine efficacy of malaria vaccine candidates before moving onto human studies.

Human Volunteers

The most relevant model is the human host itself. The establishment of the CHMI model has greatly helped malaria research. The CHMI model involves exposing healthy human volunteers to the parasite *via* infective mosquito bites, monitoring the volunteers closely for signs and symptoms of malaria infection, and treating the volunteers with drug upon detection of fever and/or detection of parasites (45, 176).

The CHMI model uses the most relevant host-parasite pair. While CHMI has been performed *via* other routes such as intravenously and intramuscularly, it is more commonly done *via* infective mosquito bites. The use of infective mosquito bites in the model mimics the natural route of infection, offering advantages in the prediction of the potential efficacy of vaccine candidate against natural infections. However, it also has its limitations. The CHMI model is often performed with one parasite strain, while there are many antigenically diverse heterologous parasites in the field. Infection in the CHMI model is controlled and the parasite load is administered at one single time, whereas high parasite load at one single time is uncommon in natural field setting. Despite the limitations, CHMI studies with no observed efficacy could halt the pursuit of large and expensive clinical trials in malaria-endemic areas in time. CHMI studies with partial efficacy could provide insights on how protective efficacy could be improved by alterations in

vaccination regimes such as the number of doses and number of immunizations. In particular, through a series of CHMI studies, the company Sanaria was able to optimize their PfSPZ vaccine, which is composed of radiation-attenuated, aseptic, purified, cryopreserved *P. falciparum* sporozoites, to induce sterile protection against homologous challenge for at least 59 weeks (24) and heterologous challenge for at least 33 weeks (51) in malaria-naïve individuals. The vaccination also prevented naturally transmitted heterogeneous *P. falciparum* in malaria-endemic adults in Mali for at least 24 weeks (vaccine efficacy of 29%) (14). Further CHMI studies to optimize dosage and vaccination regimes could improve the vaccine efficacy.

Immunity and Correlates of Protection Against the Pre-erythrocytic Stage

The malaria parasite has a complex life cycle. Depending on the stage of development in its mammalian host, the parasite can be extracellular or intracellular. They can also infect different cell types. Hence, various innate and adaptive immune mechanisms are needed for parasite control and elimination. In order to develop an efficacious pre-erythrocytic stage vaccine, it is important to know the protective immune mechanisms to induce (**Table 1**).

In addition, through better understanding of the mechanisms involved in the protection, we could potentially identify correlates of protection. The identification of correlates of protection is particularly important to the vaccine development as it helps to assess vaccine efficacy and design better immunogens. Through various animal models, we are beginning to tease out the potential correlates of protective immunity.

Innate Immunity

Upon infection, the innate immunity is triggered by the malaria parasites. Immune responses initiated by the innate immune system in response to parasites play key roles in protective immunity development. Early pro-inflammatory responses regulate anti-parasitic Th1 development and promote effector cell function for efficiently clearing infections. The use of a proper adjuvant is necessary to trigger the adequate innate pathway.

Cytokines

Cytokines play an important role in the protection against malaria. Upon infection, the RNA of the parasites is recognized by the cytosolic pathogen-recognition receptors of mouse hepatocytes. As a result, type I interferon pathway is induced, which can inhibit late stage parasites. Type I interferon leads to the recruitment of leukocytes that inhibit late liver forms through IFNγ (179, 212). IFNγ can inhibit the development of *P. yoelii* and *P. berghei in vitro* and *in vivo* in mice (177, 178), *P. falciparum* in human hepatocytes *in vitro* (213) and *P. vivax* infected chimpanzees *in vivo* (214). The effect of IFNγ is through the induction of the inducible nitric oxide synthase enzyme in hepatocyte which generates high of toxic nitric oxide (180–182).

In addition to IFNγ, IL6, and TNFα have also been implicated in protection. TNFα is able to inhibit parasite liver stage indirectly through induction of yet-to-be-identified mediators secreted by hepatocytes (215) or through IL-6 on non-parenchymal cells (216). IL-6 inhibits liver stage development through the induction of iNOS (217, 218).

NK and NKT Cells

NK and NKT cells are abundant in the liver, and interact with the parasites to initiate liver-stage cell-mediated immunity. Following the activation of the type I interferon pathway, the hepatocytes produce chemokines to recruit macrophages, neutrophils, lymphocytes, NK and NKT cells to the site of infection in mice (179, 219). This eventually leads to the killing of late liver stage parasites by NKT cells. NK cells have also been shown to inhibit the development of the liver stages in the hepatocytes, limiting the infection and/or reinfection in mice (185). NK cells also play an important role in CD4 T cell priming during murine malaria infections (186, 187), bridging between the innate and adaptive immunity.

γδ T Cells

Similar to NK T cells, γδ T cells are innate-like T cells that have been postulated to bridge the gap between innate and adaptive immunity (220). Early production of IFNγ by γδ T cells was detected following *in vitro* exposure of *P. falciparum*-infected RBCs to PBMCs from malaria-naïve donors (188). In mice, γδ T cells induced by whole sporozoites vaccination can inhibit intrahepatic parasitic development (190). γδ T cells are also important for the induction of early immunity against malaria (191). γδ T cell-deficient mice immunized with irradiated sporozoites were more susceptible to liver stage infection 42 h following a sporozoite challenge (191). γδ T cells can also directly prime CD4 and CD8 T cell responses *in vitro* (192, 193).

Sterile protection in mice following vaccination with irradiated sporozoites requires γδ T cells (194). Without γδ T cells, protective CD8 T cell responses were impaired (194). γδ T cells have been postulated to act either as effector cells that operate in the absence of αβ T cells, or as accessory cells for appropriate protective responses from other cells (194). In humans, γδ T cells have also been shown to recognize malaria antigens and proliferate, conferring immunity against clinical malaria in children from Uganda (221). In addition to influencing the protective CD8 T cell response, γδ T cells can also influence cytokine production. Higher frequencies and higher cytokine production by γδ T cells correlate with protection against subsequent infection in children living in endemic settings (189, 222). Recent studies on irradiated sporozoite vaccines have shown that γδ T cells expanded in a dose-dependent manner in immunized malaria-naïve subjects (6, 24), and were associated with protection (24). Hence, γδ T cells could be a potential correlate of protection, and further studies to better define a most appropriate outcome to represent a measurable positive correlation of γδ T cells with protection would be advantageous for vaccine development.

CD8α Dendritic Cells

Early adaptive immunity is triggered as early as a few hours post an infective mosquito bite, with T cell activation being observed in the skin draining lymph nodes in the murine model

TABLE 1 | Immunity against pre-erythrocytic stage parasites.

Immune response	Mode of action	References
Innate type I interferon response	*Plasmodium* RNA as a pathogen-associated molecular pattern (PAMP) to activate a type I IFN response, which in turn reduce the liver parasite load	(177–179)
Cytokines	IFNγ induces the inducible nitric oxide enzyme to produce nitric oxide to kill the sporozoites	(180–182)
	TNFα increases the capacity of monocytes and macrophages to phagocytose parasite to limit parasite infection	(183, 184)
NK cells	Inhibit liver stage development to limit liver parasite load CD4 T cell priming	(185–187)
γδ T cells	IFN-γ production Inhibit intrahepatic parasitic development Prime CD4 and CD8 T cell responses	(188–194)
CD8α dendritic cells	CD8 T cell priming	(195–197)
CD8 T cells	Lysis of infected hepatocytes by perforin and granzymes	(184, 198–200)
	Indirectly through the action of pro-inflammatory cytokines such as IFN-γ to mediate anti-parasite effects	(180–182, 201–204)
CD4 T cells	B cell development to produce antibodies against the liver stage	(198)
	Survival of protective effector and memory CD8 T cells	(205, 206)
	Can be induced to expresses CD107a, a marker for cytotoxic degranulation, to mediate protection	(48)
Antibodies	Inhibit sporozoite motility in the liver Mediate cytotoxicity against sporozoites in the host skin	(207, 208)
	Opsonize sporozoites for the subsequent sporozoite phagocytosis by monocytes or macrophages	(208, 209)
	Inhibit sporozoite invasion into hepatocytes	(210)
	Inhibit sporozoite development inside the hepatocytes	(210)
	Bind to parasite neo-antigens such as heat shock protein expressed at the surface of infected hepatocytes to induce liver parasite killing through an antibody-dependent cell-mediated mechanism	(211)

(196). After dermal inoculation, a fraction of sporozoites actively migrates to the draining lymph nodes (195). There is a direct uptake of the parasites by lymph-node resident CD8α dendritic cells followed by CD8 T cell-dendritic cell cluster formation in the draining lymph nodes (223). CD8α dendritic cells are also shown to be essential for the development of the protective immunity induced by intravenous injection of irradiated sporozoites since mice depleted of these subsets are not protected against a sporozoite challenge (224, 225). A subsequent study showed that splenic but not liver CD8α dendritic cells are the main cells involved in effector parasite specific-T cell priming (226). It was also shown recently that monocyte-derived CD11c cells infiltrated the liver after infection, acquired parasite-derived antigens and primed protective CD8 T cells (227). The role and functions of other dendritic subsets is controversial and remains to be determined (199, 228, 229).

Adaptive Immunity

As with any vaccination, the focus has been to trigger the adaptive immunity to induce an efficacious and long-lasting immunity. Various arms of the adaptive immunity are required to act in concert to provide protection against malaria.

CD8 T Cells

CD8 T cells have been implicated as the principal effector cells, central to protection against malaria. The importance of CD8 T cells in protective immunity was first demonstrated in mice vaccinated with irradiated sporozoites (177). The sterile immunity induced by the vaccination was abolished when CD8 T cells were depleted (177, 178). CD8 T cells can kill the parasites in mice (200) either directly through lysis of infected hepatocytes by perforin and granzymes (184, 230) or indirectly through IFNγ-mediated protection (180–182, 201–204). It must be stressed that while leukocytes and, in particular, CD8 T cells can kill liver parasites by these mechanisms, they differ depending of the host/parasite combinations (231).

Given the central role of CD8 T cells in protection, it is one potential correlate of protective immunity. In humans, CD8 T cells have been shown to be associated with protection from severe malaria (232), and a few of the identified CD8 T cell responses are directed against pre-erythrocytic stage antigens such as LSA1 and CSP (233, 234). However, vaccination studies, where human volunteers were immunized with irradiated sporozoites, showed that, while CD8 T cell response were also detected against various pre-erythrocytic stage antigens, the responses were not found to be associated with protection (235). More recently, a human trial where human volunteers were immunized with irradiated sporozoites showed seemingly contradicting data, where sterile protection correlated with the numbers of IFNγ-producing CD8 T cells in isolated PBMCs (6). This is also evident in animal studies on irradiated sporozoite vaccines, where high frequency of parasite-specific

CD8 T cells was observed in the liver of non-human primates and mice, and was associated with protection in mice (236). The differences are likely due to the differences in the vaccination regimes and the methods to detect/quantify T cells. Indeed, in humans, T cell activity is measured in peripheral blood, whereas, in mice, T cell activity is usually measured in spleen or liver.

Central memory T cells

Memory T cells provide long-term protection. Upon re-infection, these cells rapidly gain effector functions including cytokine production and lytic activity. There are three subsets of memory T cells: (1) central memory T cells, which predominantly reside in lymphoid tissues, (2) effector memory T cells, which reside in the spleen and peripheral tissues, and (3) tissue-resident memory T cells, which reside in the tissues and do not recirculate. The role of central memory T cells in protection against malaria is limited. While central memory T cells can produce IFN-γ after *in vitro* stimulation (201), their presence has not been associated with protection. Despite having a large proportion of central memory T cell, mice that were vaccinated with modified vaccinia ANKARA expressing the multiple epitope string and thrombospondin-related adhesion protein (ME-TRAP) were not protected from malaria challenge (237).

CD8 effector memory T cells

In contrast to the central memory T cells, the presence of effector memory T cells has been associated with sterile protection in the murine model, although large numbers are required for protection against malaria (238). Long-term sterile protection was only observed in mice when the parasite-specific CD8 T cells made up >1% of the total peripheral blood CD8 T cell population (238). Degree of protection in mice correlated with the frequencies of CD8 effector memory T cells present in liver, and failure to achieve the protective threshold frequency of these cells might make the host susceptible to infection (203).

CD8 tissue-resident memory T cells

More recently, a new subset of memory T cells, with a distinct gene expression profile, has been characterized (202). The liver tissue-resident memory T cells develop naturally during the course of an immune response following TCR stimulation, with rapidly expanding population due to the liver infection or inflammation in mice (239). These cells are found to be patrolling within the liver sinusoids, a process dependent on LFA-1–ICAM-1 interactions (240). Tissue-resident memory T cells were essential for sterile protection against sporozoite infection in mice following immunization with irradiated sporozoites (241).

CD4 T Cells

In contrast to CD8 T cells, the role of CD4 T cells in protection against malaria is not well understood. Despite this, it is clear that the development and maturation of an effective CD8T cell response is dependent on CD4 T cells help. CD4 T cells are activated to amplify the anti-pathogen response by driving B cell germinal responses and supporting CD8 T cell activation. Mouse hepatocytes express MHC Class I and Class II molecules that can be loaded with parasite antigen-derived epitopes following the TAP or the endosomal pathways (242–245). CD4 T cells are required to prime effective immunity. CD4 T cells recognizing CSP have been shown to protect against a *P. yoelii* sporozoite challenge in mice (243, 246). Pre-immunization but not pre-challenge depletion of CD4 T cells also resulted in a loss of protection in mice immunized with sporozoites, suggesting that CD4 T cells might provide signals for efficient maturation of effector CD8 T cells (247). In mice, CD4 T cells were essential to ensure survival of protective effector and memory CD8 T cell induced by irradiated sporozoites (205, 206). In humans, many studies have described a CSP-specific CD4 T cell response that is associated with protection against natural infection and disease (248), and is able to inhibit pre-erythrocytic stage development (249). CD4 T cells have also been shown to correlate with sterile protection in humans following immunization with live sporozoites under chloroquine prophylaxis. CSP-specific CD4 T cells were induced to express CD107a, a marker for cytotoxic degranulation, after immunizations with live sporozoites under chloroquine prophylaxis in humans, and these cytotoxic markers has been shown to be associated with sterile protection against the pre-erythrocytic stages (48). In addition to being crucial for B cell development to produce antibodies, CD4 T cells are also important for CD8 T cell responses.

While it is clear that CD4 T cells are involved in protective immunity against malaria, its use as a potential correlate of protection needs further validation. Together with CD8 T cells, the definitive role of CD4 T cells in protection requires more unraveling and the information will be critical to the development of a validated T cell-based correlate of protection for vaccine efficacy assessment.

Antibodies

Lastly, in addition to inducing an effective CD8 T cell response, the development of many malaria candidate vaccines also aims at being able to induce an effective antibody response. Antibodies are often the first host immune response being studied. Antibodies against the pre-erythrocytic stage can mediate protection by limiting pre-erythrocytic stage infection and development. More specifically, the antibodies do so by (1) inhibiting sporozoite motility in the dermis and liver (207), (2) mediating cytotoxicity against sporozoites in the host skin (208, 250), (3) opsonizing the sporozoites and subsequently facilitating sporozoite phagocytosis by monocytes or macrophages in the spleen or the liver (208, 209), (4) inhibiting sporozoite invasion into hepatocytes (210), (5) inhibiting sporozoite development inside the hepatocytes (210), and (6) binding to parasite neo-antigens such as heat shock protein expressed at the surface of infected hepatocytes and eventually inducing liver parasite killing through an antibody-dependent cell-mediated mechanism that is likely to involve Kupffer cells or NK cells (211).

Antibodies are potential correlates of protection. Passive transfer of RTS,S-induced human anti-CSP monoclonal antibodies into humanized mice at concentrations within the range observed in human, protected the mice against *P. falciparum* challenge (170). Immunization with genetically-attenuated sporozoites that arrest late in the liver stage development elicited protection against both a sporozoite

challenge and a direct blood stage challenge by inducing the production of stage-transcending protective antibodies in mice (251). Sporozoite-specific antibodies induced by vaccination with irradiated sporozoites (252) or genetically-attenuated sporozoites (253) have also been shown to inhibit sporozoite invasion into human hepatocytes *in vitro* and correlate with protection in human individuals (254–256). However, a recent human study examining protection following vaccination with irradiated sporozoites in malaria-naïve individuals has found no significant correlation of antibody response with protection (46). In addition, there is no distinct antibody profile that allows differentiation of protected individuals from the susceptible individuals (257) following RTS,S vaccination. There is increasing awareness that, in addition of high level of antibodies, the quality of the antibodies is also important. *In vitro* assays to examine the functionality of the induced antibodies following vaccination of pre-erythrocytic stage vaccine have been developed. These assays include gliding motility assays (258), sporozoite traversal and invasion inhibition assays (6, 253, 259), and pre-erythrocytic stage development inhibition assays (136, 260). Recently, human monoclonal antibodies have been derived from volunteers immunized with irradiated sporozoites. These antibodies recognized an important epitope at the junction of the N terminal part and the repeat regions of the CSP. This can lead to the design of better CSP-based vaccines (146, 261). Further studies to draw parallels between the readouts of these assays and the protection in the field are necessary to develop a validated antibody-based correlate of protection for vaccine efficacy assessment.

CONCLUDING REMARKS

In contrast to the limited efficacy of RTS,S and other subunit vaccines, vaccination with sporozoites has had more success. In addition to promising efficacy data, a series of recent clinical trials on sporozoite-based vaccines has demonstrated formidable advances in overcoming issues in vaccine manufacturing and delivery (6, 14, 23, 46). Furthermore, there are now data showing that sporozoite vaccines are safe and tolerated in malaria-endemic areas (14, 23). While the vaccine efficacy is markedly reduced against heterologous CHMI (as compared with homologous CHMI), it is encouraging that it offers some protection against heterologous CHMI (25). Further studies to optimize the immunization regimen could potentially improve the vaccine efficacy. Here, we reviewed the various types of vaccination strategies with sporozoites and the different animal models being used for the vaccination studies. We also discussed the mechanisms of protection against the pre-erythrocytic parasites. While the mechanisms of protection are slowly being unraveled, the establishment of validated correlates of protection for assessment of vaccine efficacy has proved to be challenging. Half of the world population is at risk of a malaria infection. The target population is highly diverse, with individuals from different age groups (infants, adults and elderly), different exposed status (endemic and non-endemic), and different immunological background (immunocompromised and pregnant). The presence of co-infections in some populations in malaria-endemic regions adds further complexity. In addition, the complexity of the parasite and the diversity of its genome also makes it difficult to definitively establish correlates of protection. Depending on which part of the parasite life cycle the malaria vaccine candidates target, different forms of immunity are induced. As it is still unclear if the ultimate goal of a malaria vaccine should be to protect against infection or simply to protect against disease, different clinical endpoints have been used to measure vaccine efficacy. Vaccine-induced immune responses that correlate with protection against one endpoint may not necessarily correlate with protection against a different endpoint. Hence, until date, there is no validated correlate of protection. A concerted effort to develop/refine relevant animal models, investigate the definitive mechanisms of protection and identify validated correlates of protection would greatly help to inform critical decisions in human vaccine clinical trial, which will accelerate future progress in the development of an efficacious malaria vaccine.

AUTHOR CONTRIBUTIONS

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KILchip v1.0: A Novel *Plasmodium falciparum* Merozoite Protein Microarray to Facilitate Malaria Vaccine Candidate Prioritization

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Passive transfer studies in humans clearly demonstrated the protective role of IgG antibodies against malaria. Identifying the precise parasite antigens that mediate immunity is essential for vaccine design, but has proved difficult. Completion of the *Plasmodium falciparum* genome revealed thousands of potential vaccine candidates, but a significant bottleneck remains in their validation and prioritization for further evaluation in clinical trials. Focusing initially on the *Plasmodium falciparum* merozoite proteome, we used peer-reviewed publications, multiple proteomic and bioinformatic approaches, to select and prioritize potential immune targets. We expressed 109 *P. falciparum* recombinant proteins, the majority of which were obtained using a mammalian expression system that has been shown to produce biologically functional extracellular proteins, and used them to create KILchip v1.0: a novel protein microarray to facilitate high-throughput multiplexed antibody detection from individual samples.

The microarray assay was highly specific; antibodies against *P. falciparum* proteins were detected exclusively in sera from malaria-exposed but not malaria-naïve individuals. The intensity of antibody reactivity varied as expected from strong to weak across well-studied antigens such as AMA1 and RH5 (Kruskal–Wallis H test for trend: $p < 0.0001$). The inter-assay and intra-assay variability was minimal, with reproducible results obtained in re-assays using the same chip over a duration of 3 months. Antibodies quantified using the multiplexed format in KILchip v1.0 were highly correlated with those measured in

the gold-standard monoplex ELISA [median (range) Spearman's R of 0.84 (0.65–0.95)]. KILchip v1.0 is a robust, scalable and adaptable protein microarray that has broad applicability to studies of naturally acquired immunity against malaria by providing a standardized tool for the detection of antibody correlates of protection. It will facilitate rapid high-throughput validation and prioritization of potential *Plasmodium falciparum* merozoite-stage antigens paving the way for urgently needed clinical trials for the next generation of malaria vaccines.

Keywords: *Plasmodium falciparum*, merozoite, antibodies, vaccine candidates, protein microarray, bioinformatics

INTRODUCTION

Protein microarrays are increasingly used in the "omic" era of research in multiple formats that share the basic requirement to investigate interactions of tens to thousands of proteins simultaneously (1). They have had important translational applications in biomarker discovery to guide patient diagnosis, treatment and prognosis, as well as in drug discovery and vaccine antigen identification (2). Protein microarrays have facilitated a rapid, systematic and high-throughput approach to probing an entire pathogens' proteome or fraction thereof for immunoreactivity, in an approach that forms part of a reverse vaccinology workflow. These have aided in the discovery of potential diagnostic markers for *Mycobacterium tuberculosis* and *SARS-coronavirus* as well as potential vaccine candidates in over 30 human pathogens including *Plasmodium falciparum* (2, 3).

P. falciparum malaria causes ∼450,000 deaths per year (4), and is of major public health importance to sub-Saharan Africa (5). Recent gains in reducing the burden appear to have stalled despite ongoing control efforts (4, 6). Efforts to design a highly effective vaccine that would protect against this disease have been hampered by the complexity of the organism and its' multi-stage life cycle: its genome encodes >5,300 proteins that are expressed variably in different tissues as the infection develops in the host (7). Coupled to this is an impressive array of strategies for generating protein polymorphisms or protein variants and redundant erythrocyte invasion pathways, which facilitate immune evasion (8–10). Consequently, although efforts to develop a highly effective malaria vaccine have been on-going for over a century, this goal has yet to be achieved. The current leading vaccine candidate against *P. falciparum* malaria has limited efficacy and induces only short-lived protective immunity (11, 12).

Multiple *P. falciparum* and/or *P. vivax* protein arrays have been designed over the past decade to help identify and prioritize potential malaria vaccine antigen candidates. The majority of these arrays have been manufactured using either the *E. coli*-based or the wheat germ cell free *in-vitro* transcription/translation expression system, with the largest to date including ∼30% of the entire *P. falciparum* proteome (13–17). Protein selection was based on stage-specific transcription or protein expression, sub-cellular localization, secondary protein structures or documented immunogenicity in human and animal models. However, the *in-vitro* transcription/translation systems are relatively poor at generating functional surface proteins, which frequently require disulphide bonding and/or post-translational modification to attain their correct three-dimensional structure. Nevertheless, subsequent studies have down-selected proteins from this initial panel (18–31), indicating that essentially >75% of the parasite genome has yet to be evaluated in the context of immunity. A few additional proteins have been tested independently in smaller scale studies accounting for only a marginal increase in the proportion of the parasite proteome evaluated to date (32–34). These studies have rationally selected merozoite proteins that were established or plausible targets of antibodies, and evaluated antibody associations with protection in longitudinal studies using standard ELISA-based approaches (32, 33). They highlighted the importance of evaluating a broad repertoire of antigens and combinations of antibody responses in studies of acquired immunity. However, there still remains a need for a common platform with standardized protein expression and high-throughput antibody detection methods that can be applied widely across different clinical studies (35). This would accelerate identification of protective antibody targets and facilitate the comparisons between studies and populations.

To contribute to vaccine candidate discovery, as well as the validation and prioritization of existing candidates for clinical trials, we designed a novel protein microarray. We focused on the merozoite stage that is a target of immunity that can prevent or reduce the clinical symptoms of malaria. As per the case with other infectious diseases (36, 37), we hypothesized that proteins on or associated with the surface of the invasive *P. falciparum* merozoite would be accessible targets for protective antibodies (33). We mined the literature to identify multiple potential surface-associated merozoite proteins (32–34, 38–43) and added new proteins that were identified as immunogenic in adults from malaria-endemic countries and had proteomic and/or bioinformatic features suggestive of merozoite surface-localization, secretion and/or involvement in erythrocyte invasion (44). We expressed and purified these proteins and printed them on a custom microarray, which we refer to as KILchip v1.0 for its origin at the KEMRI-Wellcome Trust Research Programme in Kilifi, Kenya where the majority of the work was carried out. We demonstrate that KILchip v1.0 is highly specific, has minimal inter- and intra-assay variation, and is strongly correlated with equivalent data acquired using the gold-standard monoplex ELISA.

MATERIALS AND METHODS

Protein Selection

We aimed to design a microarray that would include proteins already considered as vaccine candidates, as well as novel proteins that had not been studied in the context of protective immunity. This would serve multiple purposes: (i) validation of existing vaccine candidates in new sample sets, (ii) identification of novel potential candidates and (iii) facilitation of head-to-head comparisons of all selected candidates in the same experiment. To this end, we selected a panel of antigens previously published in Zenonos et al. (38), Richards et al. (32), Crosnier et al. (39), Tetteh et al. (34), Raj et al. (43), Polley et al. (40, 45), Kimbi et al. (46), Metzger et al. (47), Taylor et al. (41), and Burghaus and Holder (48), for inclusion in the KILchip v1.0. These proteins are known or predicted to be anchored or associated with the surface of merozoites, secreted from its apical organelles or involved in erythrocyte invasion and have been shown to correlate with protection from clinical malaria (32, 33). We also included 28 novel proteins selected through a protein discovery pipeline that employed proteomic approaches for the detection of proteins that were either immunogenic or located on the surface of merozoites (44). Down-selection criteria for the novel proteins included either (i) the presence of a predicted N-terminal signal peptide and/or transmembrane domain(s), (ii) upregulated transcription at the late stages of the asexual life cycle, (iii) a predicted role in merozoite invasion (49) and (iv) novel with regards to a potential role in protective immunity.

Recombinant *P. falciparum* Protein Expression

Plasmids containing codon-optimized genes of interest were either obtained from the plasmid repository Addgene (https://www.addgene.org) or newly synthesized by GeneartAG as has been previously published (38, 39). Briefly, predicted signal peptides and transmembrane domains were excluded and the serine or threonine amino acid residues in all potential N-linked glycosylation sites (NXS/T) were substituted with alanine. Codon-optimized genes of interest were sub-cloned into a derivative of the pTT3 expression vector (also obtained from Addgene) that contained an N-terminal signal peptide derived from the mouse *kappa* light chain to drive secretion of antigen and a rat Cd4 domains 3 and 4 tag followed by a hexa-histidine tag for protein purification (39). Proteins were subsequently expressed using the Expi293 expression system (Invitrogen) according to manufacturer's instructions. Briefly, Expi293F cells were cultured to a density of 2.0×10^6 cells/ml and transfected with expression vectors using the Expifectamine 293 transfection reagent (Invitrogen). Cells were then incubated at 37°C with 8% CO_2 in an orbital shaker at 125 rpm. Culture supernatants were harvested 6 days post-transfection and proteins purified using Ni-NTA purification columns (Invitrogen). The majority of proteins included in KILchip v1.0 were expressed using the above mammalian expression system.

A minority of proteins were expressed in *E. coli* using pGEX-2T and pMAL-c2X vectors to produce fusion proteins with the carriers glutathione-S-transferase (GST) and maltose-binding protein (MBP), respectively. These were transformed into BL21 (DE3) *E. coli* cells and expressed as previously described (34, 40–42). The gene encoding *Pf*SEA1 protein was amplified from *P. falciparum* 3D7 cDNA using previously described primers (43). The PCR products were cloned into pEXP5-NT/TOPO expression vector and transformed into BL21 (DE3) pLysS *E.coli* cells. Cell expansion, induction of protein expression and subsequent purification was performed as previously published (34, 40–42). Purified recombinant proteins were dialysed into phosphate buffered saline and quantified using NanoDrop (Thermo scientific) before printing onto nitrocellulose slides. Further details are provided in the **Supplementary information**.

LC-MS/MS Analysis, Protein Validation, and Circular Dichroism Spectroscopy

Five to fifteen μg of purified recombinant proteins were denatured in 50 mM Tris-HCL pH 8.0 (Sigma) containing 8 M urea (Sigma). Proteins were reduced with 40 mM dithiothreitol (Sigma), alkylated with 80 mM iodoacetamide (Sigma) and precipitated using cold acetone. Pelleted proteins were resuspended in 15 μl of 6 M urea in 50 mM Tris-HCL pH 8.0 and digested with trypsin/Lys-C mix (Promega) according to manufacturer's instructions using the two step in-solution digestion. Peptides were desalted using P10 c18 pipette ZipTips (Millipore), dried using the Speedvac concentrator (Thermo Scientific) and resuspended in 15 μl of 99% H_20, 1% acetonitrile, and 0.1% formic acid. Peptides (5 μl) were loaded using a Dionex Ultimate 3,000 nano-flow ultra-high-pressure liquid chromatography system (Thermo Scientific) on to a 75 μm × 2 cm C18 trap column (Thermo Scientific). Chromatographic separation of peptides was carried out on a reverse-phase 50 cm-long column (Thermo Scientific) maintained at 40°C over a 60-min elution gradient (2–40% of mobile phase B; 80% acetonitrile with 0.1% formic acid) at a flow rate of 0.3 μl/min. Peptides were measured using LC instrumentation consisting of a Dionex Ultimate 3,000 nano-flow ultra-high-pressure liquid chromatography system (Thermo Scientific) coupled via a nano-electrospray ion source (Thermo Scientific) to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). The ms^1 settings were: Resolution, 70,000; Automatic gain control (AGC) target, 3e6; maximum injection time, 100 ms; scan range, 380–1600 m/z; while the ms^2 settings were: Resolution, 17,500; AGC target, 5e4; maximum injection time, 100 ms; isolation window, 1.6 m/z. The top 10 most intense ions were selected for ms^2 and fragmented with higher-energy collision fragmentation using normalized collision energy of 28 and these ions were subsequently excluded for the next 20 s. Mass spectrometry raw files were searched on Proteome Discoverer software version 1.3.0.339 (Thermo Scientific) using the Mascot server (Matrix Science) using a concatenated database of human and 3D7 *Plasmodium falciparum* protein FASTA sequences. Cysteine carbamidomethylation was set as a fixed modification and deamidation of asparagine or glutamine and methionine oxidation as variable modifications. The false discovery rate (FDR) was set to 0.01 for both proteins and peptides and a maximum of two missed cleavages were allowed in the database

search. A minimum of two unique peptides for a protein was considered a positive identification.

Two proteins were selected for circular dichroism spectroscopy analysis (50). Briefly, phosphate buffer (50 mM NaH_2PO_4 pH 8.0, 0.1 M NaCl) and samples in phosphate buffer were degassed in a vacuum at room temperature and the CD spectra recorded on a J-715 spectropolarimeter (Jasco). Six accumulations were taken per protein with continuous scans taken using a 1 mm (0.1 cm) quartz cuvette, a scan rate of 50 nm/min, a band width of 1.0 nm and a resolution of 0.5 nm. The raw CD data (ellipticity θ in mDeg) were normalized for protein concentration and the number of residues yielding the mean residue ellipticity [θ] in mDeg·cm2·dmol^{-1}·res^{-1}.

Data Availability

The mass spectrometry raw files generated and analyzed in the current study have been deposited to the ProteomeXchange Consortium51 (PXD011746), via the MassIVE partner repository (MSV000083144), under the following title: KILchip v1.0 A novel *Plasmodium falciparum* merozoite protein microarray to facilitate malaria vaccine candidate prioritization. The FTP for the dataset is available here: ftp://massive.ucsd.edu/MSV000083144.

KILchip v1.0 Protein Microarray Assay

Overview

We designed our protein microarray to test 21 unique serum samples per slide with a customized barcode for slide identification. Four slides fitted into a 4 × 24 hybridization cassette (Arrayit Corporation ARYC), thus accommodating 84 samples per cassette and 336 samples per hybridization workstation (ARYC), each of which contains 4 hybridization cassettes (**Figure 1**).

Microarray Protein Map

Each slide contained 21 identical protein mini-arrays. Each mini-array had 384 features (printed spots) at a volume of 400 pl per spot. Recombinant *P. falciparum* proteins ($n = 111$) and controls ($n = 17$) were printed on each mini-array in triplicate, at the same concentration, using the same printer (Ultra Marathon micro-arrayer, ArrayJet) and printing protocol. The first control spots were Alexafluor^{647} human IgG (Jackson ImmunoResearch) that served as landmarks demarcating the four edges of each mini-array (4 spots to match the edges of each mini-array). Purified human IgG (Jackson ImmunoResearch, 1 spot), served as the second control that confirmed the activity of the secondary antibody utilized in the assay. Protein printing buffer (30% glycerol/PBS, 9 spots) was used as the third set of controls that allowed for the monitoring of background reactivity and for the detection of any potential protein carryover during printing. The last sets of controls were the CD4, MBP, and GST proteins (1 spot each) to control for any potential antibody reactivity against the tags, each of which is present in one or more of the recombinant *P. falciparum* antigens. Thus, each mini-array contained a total of 384 features: 333 derived from 111 *P. falciparum* recombinant proteins (109 proteins, 2 of which were included twice) and 51 from 17 controls, all printed in triplicate.

Microarray Printing

We optimized the concentrations for individual proteins, serum samples and the secondary antibody by checkerboard titration. Proteins were printed on nitrocellulose slides (ONCYTE SuperNOVA, GraceBio) at a concentration of 250 μg/ml using the Ultra Marathon micro-arrayer (ArrayJet) with the Inkjet printing technology and the command center 1.5.0.1 (ArrayJet). Printing was carried out at 50% relative humidity and at 18°C. As a drying step, slides were incubated overnight at 18°C in the arrayer after printing, before storage in slide boxes with dessicant at 4°C until use. A salt scan was carried out at a high photomultiplier (PMT) at the 532 nm wavelength (green channel) to verify post-printing quality and at the 635 nm wavelength (red channel) to allow visualization of the landmark spots.

Antibody Detection

Printed slides were carefully assembled onto the hybridization cassette and sealed using silicone gaskets (ARYC) to form leak-proof individual wells. We modified a published protocol for the detection of antibodies (51). Briefly, wells were washed thrice with 0.1% Tween 20/HEPES buffered saline (1.4 M NaCl, 50 mM KCl, 20 mM $CaCl_2$, 10 mM $MgCl_2$, 100 mM HEPES; HBS) followed by HBS to remove any unbound proteins. Non-specific binding to the slide surface was prevented by blocking with 200 μl of 2% BSA/0.1% Tween 20/HBS for 2 h at room temperature while rotating on a microarray hybridization station (ARYC) at 350 rpm. Wells were subsequently washed thrice and incubated overnight at 4°C with 150 μl of serum diluted 1:400 and rotating at 350 rpm on the hybridization station. Thereafter, wells were washed as described above and incubated with 150 μl of donkey anti-human IgG-Fcγ fragment specific Alexafluor^{647} for 3 h at room temperature followed by three washes. Slides were carefully disassembled from the hybridization cassettes, rinsed thrice in distilled water and dried by centrifugation at 300 g for 5 min using a combiSlide adapter (Eppendorf) and stored in slide boxes in the dark. Slides were scanned using a Genepix 4,000 B scanner coupled to the GenePix Pro & Microarray Acquisition and Analysis Software (Molecular Devices).

ELISA

A standard protocol for an indirect ELISA was performed as has been previously described (52–54). Briefly, a pre-determined concentration for each recombinant protein was either heat-treated at 80°C for 10 min or left untreated and coated overnight at 4°C on 96-microwell ELISA plates (Dynex 4HBX Immunolon). Wells were washed four times in PBST (phosphate-buffered saline/0.05 Tween 20) and blocked at room temperature with 1% skimmed milk (Marvel)/PBST for 5 h. Individual wells were washed and incubated with 100 μl of either test sera or a panel of monoclonal antibodies overnight at 4°C, before being washed four times in PBST and incubated for 3 h at room temperature with 100 μl of the respective horseradish peroxidase-conjugated secondary antibody diluted in 1%Marvel/PBST. Wells were washed four times in PBST and incubated at room temperature with 100 μl of substrate development buffer consisting of H_2O_2 and o-phenylenediamine

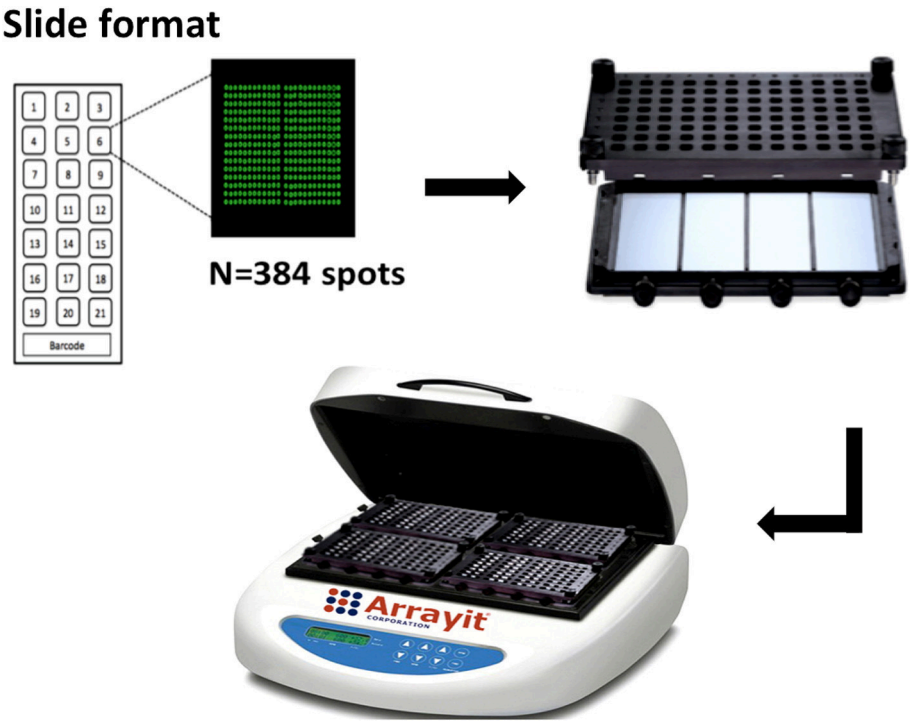

FIGURE 1 | Configuration of KILchip v1.0. Individual slides contain 21 identical mini-arrays comprised of protein spots and a barcode for identification. Each mini-array contains 384 individual spots that include proteins and controls printed in triplicate. Four slides are assembled onto a hybridization cassette, four of which are simultaneously processed in a microarray hybridization work-station (image from Arrayit Corporation, used with permission).

dihydrochloride (SigmaFAST). The reaction was stopped with 30µl of 2M H_2SO_4 per well and the absorbance read at 492 nm.

Serum Samples

Ethics Statement

This study was carried out in accordance with the recommendations of "the Declaration of Helsinki, and the Kenyan National Scientific and Ethical Review Unit (SERU)" with written informed consent from all subjects. The protocol was approved by SERU, reference number KEMRI/SERU/CGMR-C/001/3139.

Kilifi Adults

Samples from life-long adult residents of Junju in Kilifi, Kenya collected in 2008 were used for assay optimization ($n = 66$). These sera were assayed for antibody responses to well-studied recombinant merozoite proteins using monoplex ELISA assays, and the data compared with that generated from the same proteins printed on KILchip v1.0. Five serum samples from adults residing in Sweden who reported no travel to malaria-endemic regions and designated malaria naïve sera (MNS) were used as negative controls. Purified malaria immunoglobulins (MIG) were obtained from a pool of healthy Malawian adults and were used to generate a standard curve as previously described (54). An additional serum pool from Kenyan adult residents of Kilifi, Kenya was designated malaria immune sera (MIS) and served as a second positive control.

Statistical Analysis

Spot intensities were acquired using the GenePix scanner (Molecular Devices). Background, pre-scan and purification-tag intensities were subtracted (55) before analysis of within sample variability using the Coefficient of Variation (CV)

$$CV = \frac{\sigma}{\mu} \quad (1)$$

where σ is the standard deviation and μ the mean fluorescent intensity (MFI).

We used a two-step variance-stabilizing normalization to minimize the systematic variation commonly observed with this type of data. The first step was to handle the differences that could have occurred with different batches of data (machines or day) using the ComBat (SVA package in R). In the second step, variance-stabilizing normalization was used to minimize the systematic variation commonly observed with this type of data (55–58).

RESULTS

Recombinant Proteins

One hundred and ten *P. falciparum* merozoite proteins were selected from the literature based on their surface localization, known or predicted roles in erythrocyte invasion and associations with protective immunity (32–34, 38–42). Twenty-eight additional novel proteins were identified using

a combination of immuno-proteomics and bioinformatics. Of these, 111 *P. falciparum* proteins, 21 of which were novel targets, were successfully printed onto KILchip v1.0 (**Figure 2** and **Supplementary Table 1**). These included 82 full-length ectodomains or the largest predicted extracellular loop of multi-membrane proteins and 29 protein fragments obtained from different regions of eight unique proteins. Thirteen protein fragments corresponded to polymorphic variants of MSP1 ($n = 7$), MSP2 ($n = 3$), MSP3 ($n = 1$) and SURFIN4.2 ($n = 2$) (**Figure 2** and **Supplementary Table 1**). All the remaining proteins were based on the *P. falciparum* 3D7 sequence and in total, 87 unique *P. falciparum* merozoite proteins are printed on KILchip v1.0. Two antigens (MSP2-CH150/9 and PF3D7_0424400) were printed twice to serve as additional internal controls.

Protein Quality

The quality of the majority of the recombinantly expressed proteins/protein fragments included in KILchip v1.0 have been validated elsewhere, including the demonstration of specific protein-protein interactions (34, 38–41, 43, 45–48). A subset of the well-studied proteins namely: AMA1, EBA175, MSP1, MSP4, and RH5 were evaluated using monoclonal antibodies targeting conformational and disulfide-constrained epitopes in these proteins. Recombinant proteins were tested against humAbAMA1 (59), mAb R217 (60), mAb R218 (60), mAb 2.44 (61), mAb 5.2 (62), mAb 2AC7 (63, 64), and mAb QA1 (64). As shown in **Figure 3A**, each monoclonal antibody was highly reactive with its respective antigen and showed no reactivity when the target antigen was heat-denatured, confirming the presence of conformational and disulfide-constrained epitopes in the panel of recombinant proteins. As expected, low or negligible reactivity was observed between monoclonal antibodies and off-target recombinant proteins. The presence of heat-labile epitopes was further confirmed by testing native and heat-denatured recombinant proteins for their reactivity with MIS. As shown in **Figure 3B**, a decrease in immunoreactivity was observed when the proteins were heat-denatured. However, for MSP4, only a minimal drop in immunoreactivity was observed, suggesting the presence of linear epitopes. Collectively, these results suggest that proteins included in KILchip v1.0 were folded correctly and contained heat-labile, conformational epitopes. In addition, circular dichroism analysis of MSP3 and SPATR indicated that these proteins appear to be folded (**Supplementary Figure 1**).

The novel proteins reported here were expressed using Expi293F cells under the same conditions, lending support to their quality with regards to post-translational modifications and disulfide bond formation. The purity of the novel proteins was assessed using reducing SDS gels and mass-spectrometry analysis. Nineteen (90%) of the novel proteins ($n = 21$)

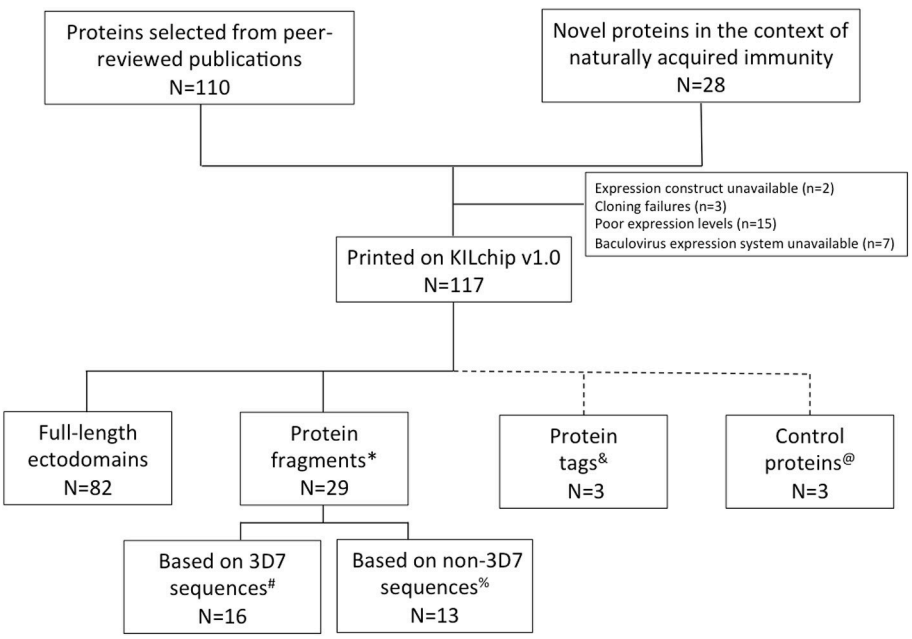

FIGURE 2 | *P. falciparum* merozoite protein panel included in KILchip v1.0. Proteins were either selected from the literature or from a combination of proteomics and bioinformatics analysis. Details of the parasite proteins are provided in **Supplementary Table 1**. *Protein fragments refers to specific amino acid regions selected from within a full-length protein ectodomain. #The protein fragments based on the 3D7 allele include MSP1-19 (Block 17), MSP1 Block 2 full, MSP1 Block 2 repeat, MSP3, MSPDBL1 N-terminus, MSPDBL1 C-terminus, MSPDBL2 N-terminus, MSPDBL2 C-terminus, 2 extracellular loops of PF3D7_0629500, *Pf*SEA1, SURFIN 4.2 3D7A, SURFIN 4.2 3D7B and the C-terminus of SURFIN 4.2. %Thirteen polymorphic variants of the *P. falciparum* merozoite proteins MSP1, MSP2, MSP3, and SURFIN 4.2 were included in KILchip v1.0. These included MSP1 Block 2 from the K1, MAD20, PaloAlto, Wellcome and RO33 alleles, the CH150/9 and DD2 alleles of MSP2, the K1 allele of MSP3 and the K1A and K1B alleles of SURFIN 4.2. &Protein tags refer to specific amino acids or polypeptides fused to target proteins to facilitate their subsequent affinity purification. These include the CD4 hexa-histidine, MBP and GST tags. @Technical controls for the assay included Alexafluor^{647} human IgG, purified human IgG and protein printing buffer.

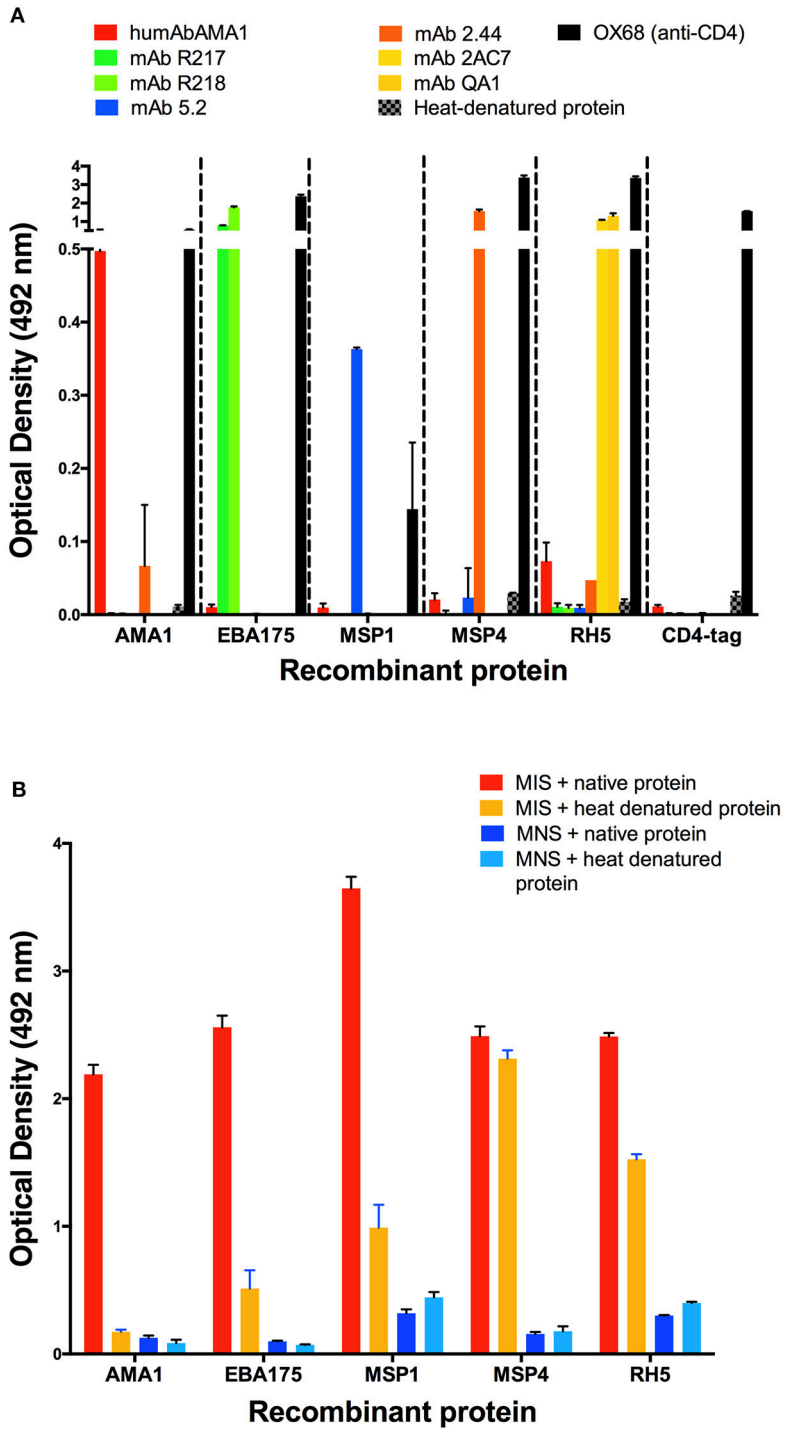

FIGURE 3 | Detection of heat-labile and conformational epitopes in a subset of recombinant proteins. Native and heat-denatured recombinant proteins were tested for reactivity against malaria immune sera (MIS), malaria naïve sera (MNS) and 5 monoclonal antibodies targeting conformational-dependent epitopes. **(A)** The monoclonal antibodies targeting conformational and disulfide-constrained epitopes on AMA1 (humAbAMA1), the F2 domain of EBA175 region II (mAb R217), the F1 domain of EBA175 region II (mAb R218), MSP1 (mAb 5.2), MSP4 (mAb 2.44), RH5 (mAb QA1 and mAb 2AC7) and rat CD4 domain (OX68) were used to measure reactivity against recombinant proteins by ELISA. All recombinant proteins demonstrated high reactivity with their respective monoclonal antibodies only. Low/negligible reactivity was observed after heat-denaturation of the recombinant proteins. **(B)** High antibody reactivity was detected with native recombinant protein. Decreased reactivity was observed with the heat-denatured proteins. Low reactivity was detected with malaria naïve sera (MNS) in both native and heat-denatured proteins.

included in KILchip v1.0 were readily detectable on Coomassie stained SDS gels and migrated at a size compatible with their predicted molecular weights (**Supplementary Figure 2**). Mass-spectrometry analysis confirmed the identity of the majority of recombinant proteins (**Supplementary Table 2**).

KILchip v1.0

Specificity

To confirm the specificity of antibody detection, individual mini-arrays were probed with the following positive and negative controls: (i) malaria immune sera (MIS), (ii) malaria naïve sera (MNS) and (iii) sample buffer consisting of 2% BSA/0.1% Tween 20/HBS. As expected, strong fluorescence was detected on each mini-array for the landmarks (blue) and commercial human IgG (green), whilst none was detected against the printing buffer (yellow) (**Figure 4A**, all panels). When probed with MIS, high levels of antibody reactivity against multiple *P. falciparum* proteins were clearly visible (**Figure 4A**, panel 1). In contrast, when probed with MNS, negligible antibody reactivity was observed. Similarly, when probed with sample buffer, nil or minimal reactivity was observed against *P. falciparum* proteins (**Figure 4A**, panel 2 and 3 respectively).

We further explored the specificity by comparing antibody reactivity to selected, previously characterized proteins in individual samples from Kenyan adults ($n = 66$) and malaria naïve Swedes ($n = 5$). The intensity of antibody reactivity varied between these selected proteins in the order AMA1 > MSP2 > MSP3 > *Pf*RH5 > RIPR (**Figure 4B** and **Supplementary Figure 3**). The median (minimum-maximum) fluorescence intensity (MFI) responses to AMA1, MSP2, and MSP3 were 23629 (1426-64840), 4762 (733-37025), and 2105 (72-4069), respectively in the Kenyan adults. MFI responses to RH5 and RIPR were low at 44 (4-914) and 28 (-2-994), respectively (**Figure 4B**). In contrast, reactivity against all *P. falciparum* proteins was low or negligible when probed with the non-malaria exposed serum from Swedish adults (**Figure 4C** and **Supplementary Figure 3**). AMA1, MSP2, and MSP3 are well-characterized immunodominant merozoite antigens while RH5 and RIPR appear to not be primary targets of naturally acquired antibody responses in some studies (32, 33, 65, 66).

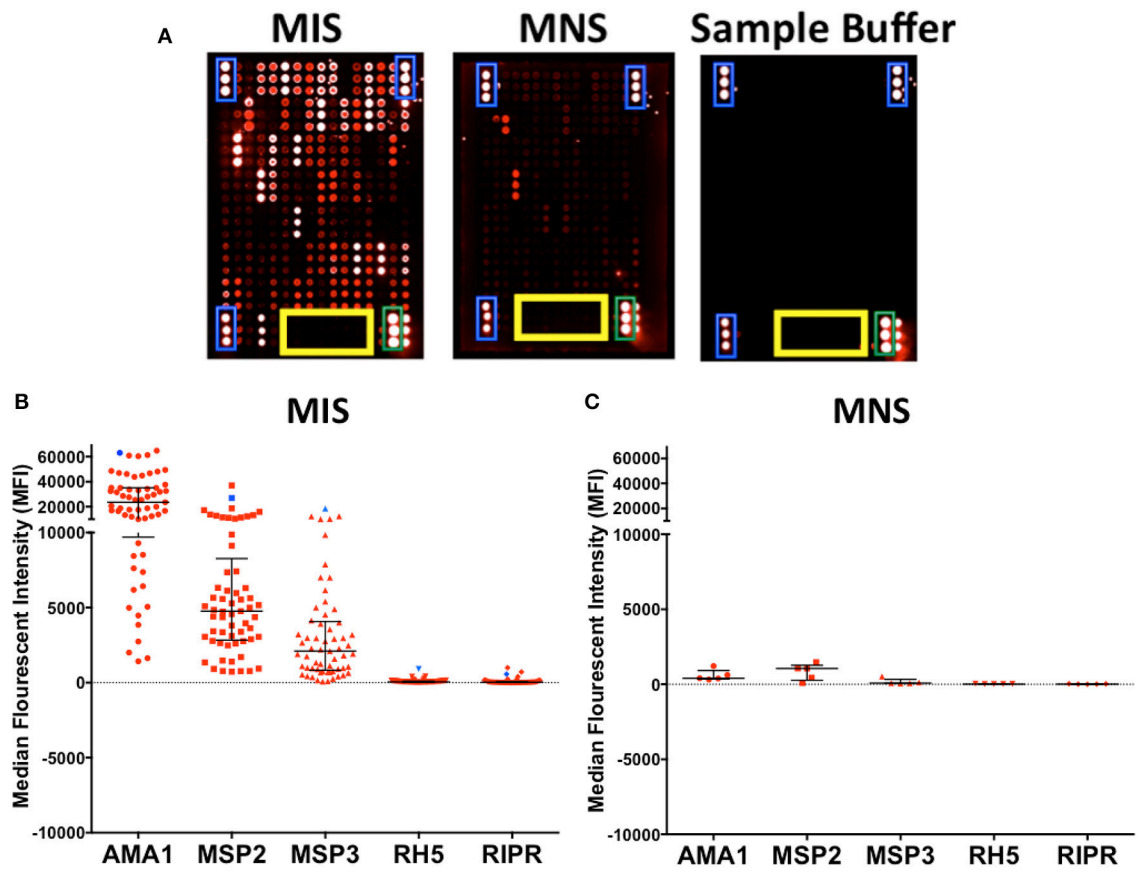

FIGURE 4 | Specificity of KILchip v1.0. Positive and negative controls were used to confirm specificity. **(A)** High antibody reactivity was detected using malaria immune sera (MIS) and low or negligible reactivity was observed with malaria naïve sera (MNS). No reactivity was observed with the sample buffer with the exception of the technical controls. Blue, landmarks; green, commercial human IgG and yellow, printing buffer. **(B)** Antibody reactivity to selected well-characterized merozoite antigens AMA1, MSP2 (3D7), MSP3 (3D7), Rh5, and RIPR varied as expected in MIS (blue) and serum samples from adult residents of Kilifi. Kenya (red). High reactivity to AMA1, MSP2 (3D7), and MSP3 (3D7) and low reactivity to Rh5 and RIPR was observed in the serum samples. **(C)** Antibody reactivity to well-characterized antigens AMA1, MSP2, MSP3, Rh5, and RIPR were all low/negligible when tested in serum samples from Swedish residents.

A direct comparison of antibody responses to multiple antigens can be evaluated using KILchip v1.0 as each protein was printed at the same concentration (250 μg/ml), and would be tested simultaneously with the same sera, providing information on the relative immunogenicity of this panel of merozoite antigens (**Supplementary Figure 4**).

Intra- and Inter-assay Variability

Intra-assay variability was tested using 66 serum samples obtained from Kenyan adults resident in Kilifi. Antibody measurements between protein spot replicates tested in the same assay and on the same day were strongly positively correlated, Spearman's R > 0.9770, $p < 0.0001$ (**Figure 5**). Inter-assay variability was tested using the positive and negative control sera. Antibody reactivity was measured repeatedly on five separate microarray slides printed over five consecutive days using identical printing conditions. As shown in **Figure 6**, the average signal intensities against individual proteins were highly reproducible.

Protein Stability on KILchip v1.0

To determine the durability of the microarray slides, we measured responses to the same batch of slides over a 3 months period using the reference reagent, MIG. These slides were printed on the same day, with identical printing conditions and

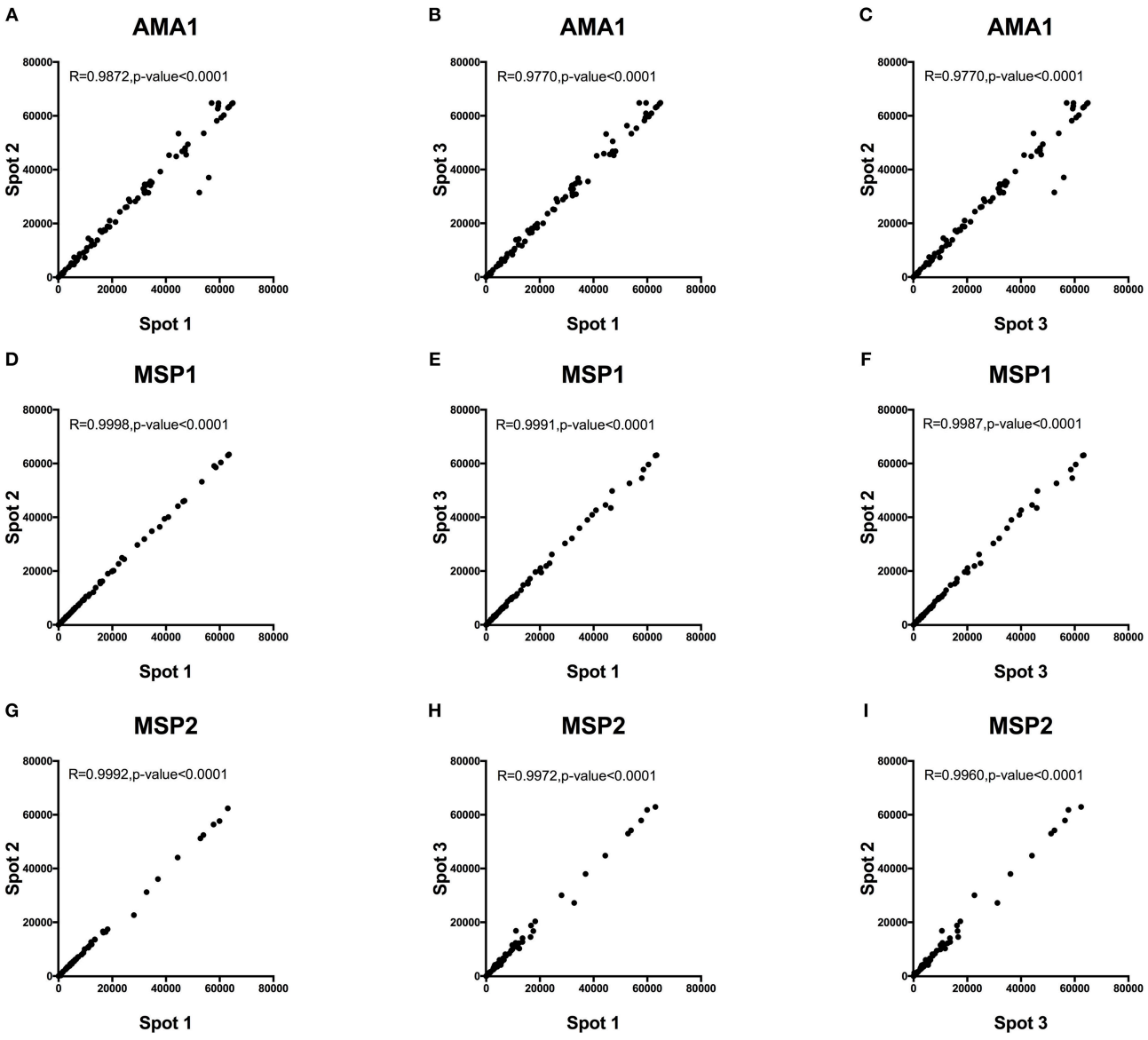

FIGURE 5 | Intra-assay variability of antibody detection by KILchip v1.0. A comparison of antibody responses to triplicate readings of AMA1, MSP1, and MSP2 was measured in 66 serum samples from adults living in the malaria endemic region of Kilifi, Kenya. A strong positive correlation of >0.98 was observed in all three-way scatter plots tested for the three antigens. Correlation between triplicate readings for recombinant AMA1 **(A–C)**, MSP1 **(D–F)** and MSP2 **(G–I)**.

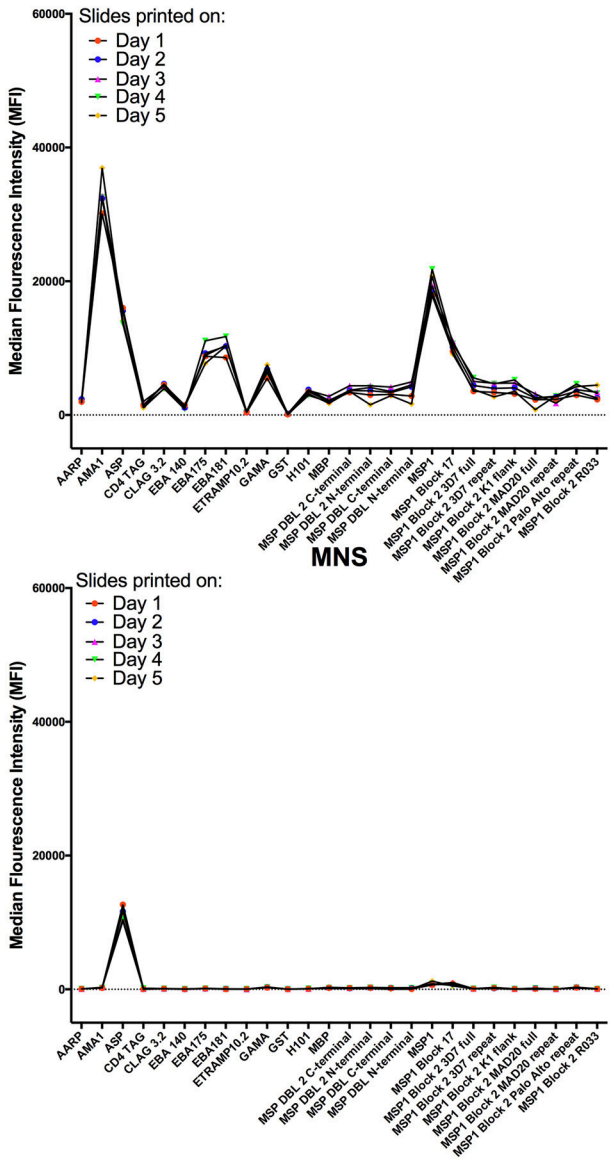

FIGURE 6 | Inter-assay variability of antibody detection by KILchip v1.0. Positive and negative controls were used to measure antibody responses to 5 protein arrays printed on 5 consecutive days using identical printing conditions and the same batch of recombinant proteins. Data presented are the mean MFI values of triplicate readings obtained with MIS (top graph) and MNS (bottom graph) to a subset of the proteins ($n = 26$) printed on each mini-array. Individual proteins are represented as dots and lines have been included to aid visualization.

the same batch of recombinant proteins. A tripling dilution of MIG was used to measure responses to each protein and to generate a standard curve. We performed a pairwise comparison of the slopes for each of the sigmoidal 5-parameter curves generated for each antigen over the 3-months period ($n =$ 4 sigmoidal curves; 6 pairwise comparisons per antigen) (67, 68). We observed no significant differences in the slopes for all 6 pairwise comparisons for 79/111 (71%) proteins (*T*-test; $p > 0.05$). Of the 32 proteins whose slopes differed significantly from each other, eight proteins had a single curve that differed while 24 proteins had 2 or more significantly different slopes. Results from 12/111 (10%) of the proteins printed on the array are shown in **Figure 7** and demonstrate that the curves to the majority of proteins were comparable indicating the detection of responses up to 3 months post printing without significant variation.

Strong Correlations Between KILchip v1.0 and the Monoplex ELISA

We compared measurements obtained using 12/111 proteins (representing ∼10% of the *P. falciparum* proteins included in the array) printed on KILchip v1.0 vs. the identical proteins using our standardized ELISA in 66 samples from Kenyan adults. As shown in **Figures 8A–L**, a strong positive Spearman's correlation coefficient R of between 0.65 and 0.95 was observed between antibodies measured by KILchip v1.0 and by the standard ELISA, $P < 0.0001$. Sixty-seven percent of the proteins tested had a correlation coefficient R above 0.8 (**Figures 8A–H**). A wider dynamic range of antibody measurement was evident in the protein array assay as observed for AMA1 (**Figure 8B**), where a subset of samples whose MFI values ranged from 40,000 to 60,000 all had an optical density value of 4.0 (red box), the upper limit detectable by ELISA.

DISCUSSION

KILchip v1.0 is a new protein microarray designed to simultaneously quantify antibodies against multiple *P. falciparum* merozoite proteins and is currently configured to include >100 proteins, the majority of which are full-length ectodomains from secreted and surface exposed proteins. This facilitates the standardized measurement of antibodies against multiple merozoite proteins in cohort studies or in controlled human malaria infections, both of which are currently used to identify and prioritize potential vaccine candidates. It can be utilized to evaluate antibody dynamics and to monitor antibody decay or longevity. The chip is flexible and can be adapted to include fewer or more proteins, allelic variants of selected proteins, full-length proteins or functionally relevant domains of proteins of interest. Adaptations of the chip could expand from the current species-specific focus on *P. falciparum* in KILchip v1.0, and be designed to test additional *Plasmodium* species singly or in parallel. Of particular importance to the research setting in sub-Saharan Africa, the chip is durable, easily stored and transported, and can be shared between partnering laboratories. With modest investments, this tool could transform the pace at which *P. falciparum* antibody response data is generated across the African continent and the same principles could be applied to other infections, ultimately contributing to improvements in health through the development of diagnostics and vaccines.

Although other protein microarrays have been in existence since the early 2,000 s, none has been designed and developed from Africa specifically to study naturally acquired immunity

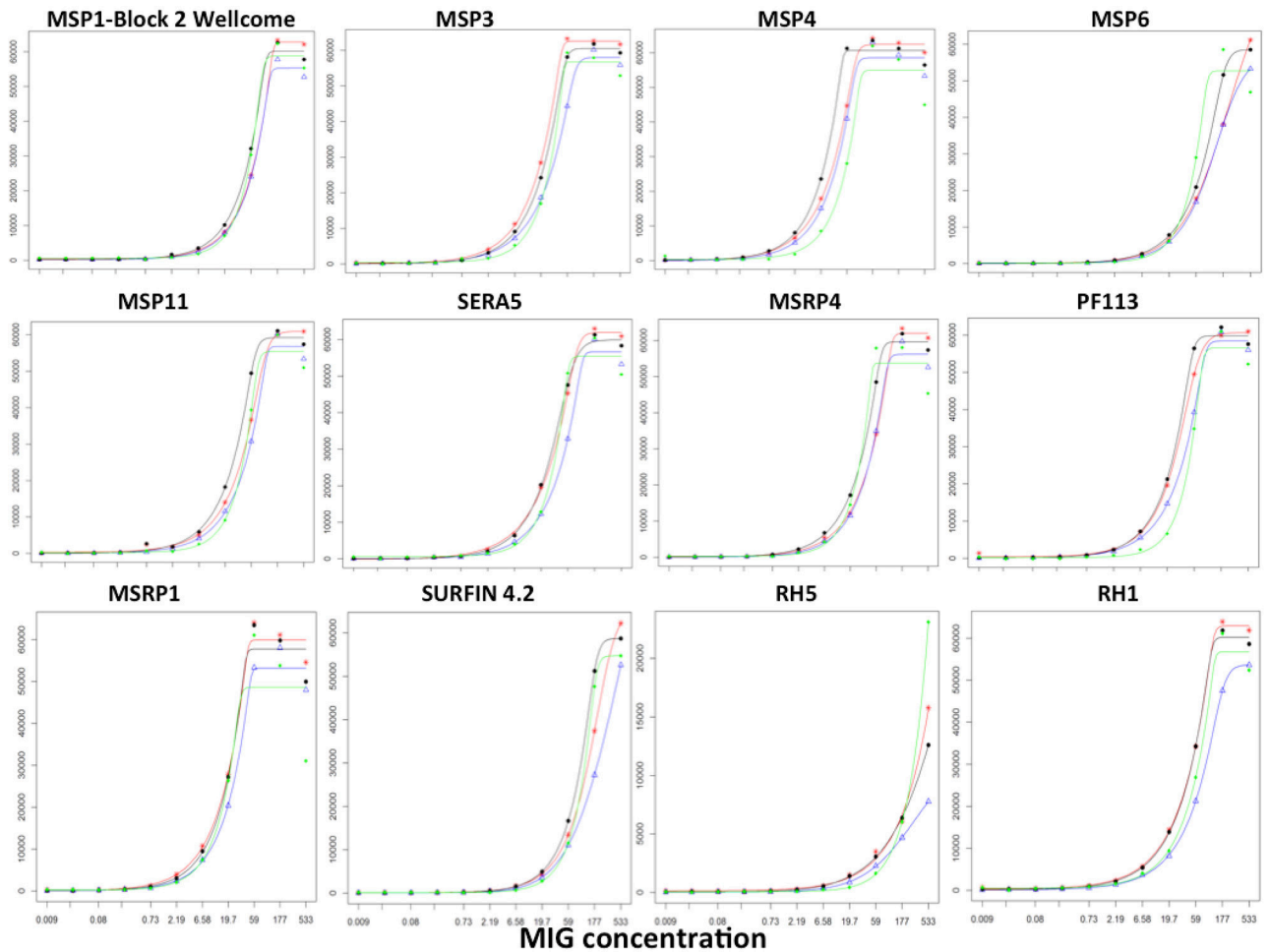

FIGURE 7 | Stability of KILchip v1.0. A reference reagent, MIG, consisting of purified immunoglobulins (98% Total IgG) was used to generate a standard curve by testing a tripling dilution against all antigens printed on the protein array. A batch of slides printed at the same time was tested 24, 40, 54, and 86 days post printing. The standard curve showed good concordance without significant variation. Red, blue, black, and green lines were obtained from measurements obtained on day 24, day 40, day 54, and day 86 post printing of the slides.

against *P. falciparum* malaria merozoites (13, 15, 69, 70). In addition, KILchip v1.0 consists predominantly of merozoite specific proteins, the majority of which were full-length ectodomains, a considerable improvement from evaluating segments of proteins, as is the case for other pre-existing protein microarrays available for *P. falciparum*. This was enabled by the use of a recently published method for transient protein expression of *P. falciparum* surface proteins in mammalian cells (39). Many of the proteins included in KILchip v1.0 are known to be immunogenic to varying degrees during natural exposure to malaria parasites, but have only been evaluated in a handful of cohort studies (32, 33). Typically, the exact nature, size and quality of antigens tested in these studies vary considerably, making it difficult to accurately compare results (35). Consequently, although some antigens have been studied for nearly 30 years, it is still not clear which proteins should be prioritized as vaccine candidates in clinical trials. KILchip v1.0 now provides a standardized tool that enables head-to-head comparisons of the immunoreactivity of a large number of proteins in multiple cohorts. One such study is already underway in which samples from at least 15 distinct geographical locations spread across 7 African countries have been tested using KILchip v1.0 (SMART: South-South Malaria Antigen Research Partnership) (71) and highlights the potential strength of KILchip v1.0.

We demonstrate that KILchip v1.0 is a sensitive tool for the detection of *P. falciparum* specific antibody responses. It is highly reproducible within and between assays and a strong concordance with the gold-standard monoplex ELISA was observed, comparable to the results reported for the different *P. falciparum* microarrays currently in use (13). Crucially, antibody measurements were stable on KILchip v1.0 up to 3 months post-printing, providing a suitable time frame for the testing of multiple samples. This is probably due to the addition of 60% glycerol to the recombinant proteins at the time of printing which

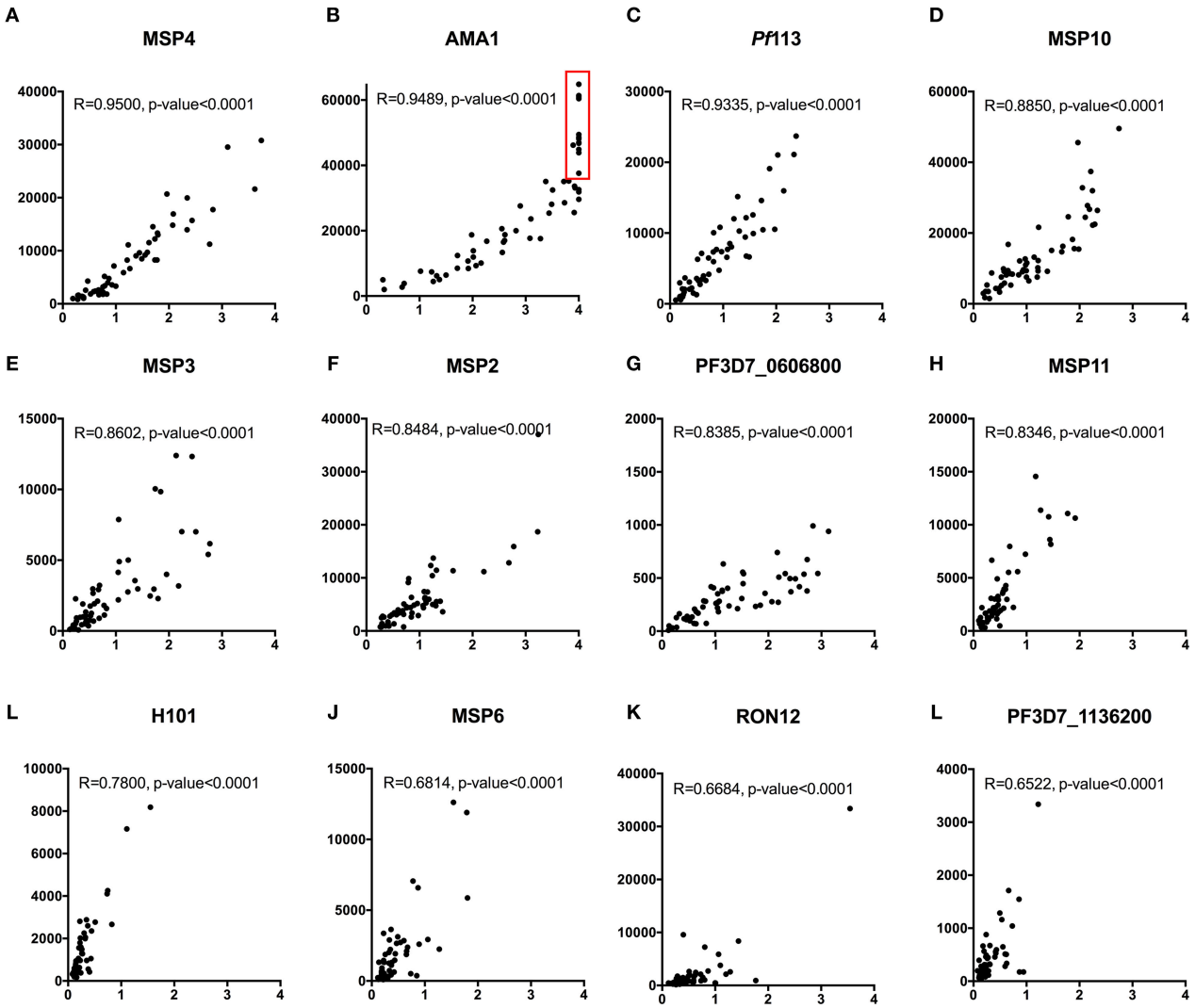

FIGURE 8 | Concordance between KILchip v1.0 and the gold standard ELISA. A comparison of antibody responses to 12 antigens measured simultaneously in KILchip v1.0 and individually by ELISA. Sixty-six serum samples from adults living in the malaria endemic region of Kilifi, Kenya were used to facilitate comparisons. A strong positive correlation of >0.8 was observed in 8 of the antigens **(A–H)** while four antigens **(I–L)** showed a correlation coefficient between 0.65 and 0.78. Red box - serum samples in which antibody responses reached the upper limit of detection when measured by ELISA, yet distinguishable by microarray with MFI values ranging from 40,000 to 60,000.

provides a stabilizing effect on proteins preventing degradation (72). Slides are conveniently stored with desiccant at 4°C.

In comparison to our standard ELISA protocol (52, 54), our microarray utilized 0.375 µl of serum to measure responses to all proteins simultaneously, compared to 23 µl required for ELISA assays of the same number of proteins. Similarly, over 100-fold less recombinant protein was required and the laser scanning and data acquisition by the GenePix 4,000 B scanner allowed for much wider dynamic ranges of antibody measurement. Lastly, this is a custom microarray format that could be scaled upwards or downwards and can be adapted to meet specific requirements. In the current version of KILchip v1.0, a single slide has 21 mini-arrays each containing 384 protein spots. This can be re-designed to include more or less proteins, to focus on allelic versions of specific proteins or to facilitate the simultaneous characterization of stage or species-specific antibody responses.

Although we selected 138 proteins/protein fragments for inclusion in KILchip v1.0, we were unable to obtain recombinant protein for 27 targets due to challenges in protein expression. These include targets such as RH4, RAP1-2, RhopH3, GLURP, SERA1, and SERA6 that have also been reported to be difficult to obtain in recombinant form in previous studies (38, 39). Also, the DBL domains of MSPDBL1 and MSPDBL2 have been previously obtained in soluble recombinant form using the baculovirus expression system but this has not yet been established within our laboratories (34). We also observed low antibody responses to some of the merozoite proteins

such as RH5 and RIPR, similar to responses measured in adults from Kenya (65) and Mali (66). However, a higher response to these proteins expressed in the wheat germ cell-free expression system has been reported in children in Papua New Guinea (73), suggesting that the protein expression system used may influence the antibody responses measured. Another limitation of the KILchip v1.0 is that the majority of proteins are based on the 3D7 *P. falciparum* isolate which may underestimate responses to highly polymorphic proteins and limit the evaluation of antigenic diversity on humoral responses and immunity. Efforts are underway to generate an additional protein array that will include allelic variants of specific proteins that warrant further study. Lastly, adsorption of proteins onto nitrocellulose-coated slides may interfere with protein structure and consequently with the detection of conformation-dependent antibody responses (74). However, these and other solid-surface platforms such as in ELISAs are widely utilized for antibody detection for vaccine candidate discovery and prioritization in multiple infectious and non-infectious diseases.

Current and future versions of KILchip v1.0 will be essential to multi-center prospective cohort studies designed to identify correlates of protection, allowing the research community to rapidly compare results head-to-head, and fast track the prioritization of new and old potential vaccine candidates. This would bridge an important gap for the urgently needed evidence base that could guide the development of the next generation of malaria vaccines. Efforts are underway to make this a resource that could be provided at a minimal cost to the malaria research community.

AUTHOR CONTRIBUTIONS

FO, JR, and KevM conceived the study. GK and JT designed the array. GK, JT, RK, MC, SH, JN, CK, MR, RF, RY, EC, TC, JM, and FG performed the experiments, GK wrote the paper with contributions from JT, KenM, NK, and FO. SK, LM, and PB provided helpful discussions. AF, KT, JB, and DC provided reagents for the array design and testing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02866/full#supplementary-material

Supplementary Table 1 | List of P. falciparum merozoite proteins included in KILchip v1.0. The details of the P. falciparum merozoite proteins provided include the expression system used, the region of the protein expressed, the P. falciparum strain the protein was based on and the recombinant expression levels achieved. The expression levels were categorized as low, intermediate and high based on the concentration of purified protein obtained following transfection of a standard concentration of 50.0×10^6 Expi293F cells. The protein concentration following purification and concentration of low, intermediate and high expressors were $<100\,\mu$g/ml, 100–250 μg/ml, and $>250\,\mu$g/ml, respectively. The expression levels are given as guide only given the variability that may be observed with different expression batches. We observed no differences in the background signals when malaria naïve sera were tested against recombinant proteins grouped according to their expression levels (Kruskal-Wallis H Test for trend: p-value = 0.5975) (data not shown).

Supplementary Table 2 | Mass-spectrometry confirmation of novel Plasmodium falciparum recombinant proteins. Mass-spectrometry confirmation of the novel Plasmodium falciparum recombinant proteins included in KILchip v1.0. Data for PF3D7_0830500, PF3D7_1025300, PF3D7_1229300, PF3D7_1252300, PF3D7_1237900, PF3D7_1343700, and PF3D7_0629500_SEG2 are not yet available.

Supplementary Figure 1 | Circular dichroism spectroscopy on recombinant SPATR and MSP3. Circular Dichroism (CD) spectra graphs for SPATR (A) and MSP3 (B) proteins are plotted from mean residual ellipticity [θ] (mDeg ·

cm2·dmol−1·res−1) as a function of the wavelength, λ (nM). The ellipticity for the blank (phosphate buffer (50mM NaH2PO4 pH 8.0, 0.1M NaCl)) was subtracted from the ellipticity for the protein samples before calculation of the mean residue ellipticity [θ].

Supplementary Figure 2 | Purity of novel Plasmodium falciparum proteins. Coomassie stained SDS gels showing 19 purified recombinant proteins. The numbers in bracket indicate the predicted molecular masses (in KDa) for each protein. Protein bands for PF3D7_1343700 and PF3D7_0629500_SEG2 were not readily visible on a coomassie stained SDS gel. Protein bands for AMA1 and CD4-hexa-histidine tags were included as controls.

Supplementary Figure 3 | Antibody responses to previously characterized merozoite proteins. The protein spots corresponding to previously characterized merozoite antigens AMA1, MSP2 (3D7), MSP3 (3D7), Rh5, and RIPR are shown in protein arrays probed with MIS and MNS sera. Red-AMA1, Yellow-MSP2, Blue-MSP3, Orange-RH5, and Green-RIPR.

Supplementary Figure 4 | Immunoreactivity of all the recombinant proteins printed on KILchip v1.0 measured using a pool of sera from naturally exposed adults. The immunoreactivity of the 111 novel proteins was tested using malaria immune sera (MIS, red bars) and malaria naïve sera (MNS, blue bars). Bar charts show mean plus standard deviation; $n = 2$.

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Functional Comparison of Blood-Stage *Plasmodium falciparum* Malaria Vaccine Candidate Antigens

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The malaria genome encodes over 5,000 proteins and many of these have also been proposed to be potential vaccine candidates, although few of these have been tested clinically. RH5 is one of the leading blood-stage *Plasmodium falciparum* malaria vaccine antigens and Phase I/II clinical trials of vaccines containing this antigen are currently underway. Its likely mechanism of action is to elicit antibodies that can neutralize merozoites by blocking their invasion of red blood cells (RBC). However, many other antigens could also elicit neutralizing antibodies against the merozoite, and most of these have never been compared directly to RH5. The objective of this study was to compare a range of blood-stage antigens to RH5, to identify any antigens that outperform or synergize with anti-RH5 antibodies. We selected 55 gene products, covering 15 candidate antigens that have been described in the literature and 40 genes selected on the basis of bioinformatics functional prediction. We were able to make 20 protein-in-adjuvant vaccines from the original selection. Of these, S-antigen and CyRPA robustly elicited antibodies with neutralizing properties. Anti-CyRPA IgG generally showed additive GIA with anti-RH5 IgG, although high levels of anti-CyRPA-specific rabbit polyclonal IgG were required to achieve 50% GIA. Our data suggest that further vaccine antigen screening efforts are required to identify a second merozoite target with similar antibody-susceptibility to RH5.

Keywords: malaria, vaccine, blood-stage malaria, antigen, merozoite, RH5, S-antigen, AARP

INTRODUCTION

Development of a vaccine against *Plasmodium falciparum* malaria is a key global health priority (1). RTS,S/AS01, the leading subunit malaria vaccine, targets the pre-erythrocytic stage of the *P. falciparum* parasite's lifecycle to deliver a short interval of moderate protection against malaria (2), which then wanes to yield 7 year efficacy of $< 10\%$ (3). Blood-stage vaccines aim to target the subsequent disease-causing stage of the *Plasmodium* life cycle and have the possibility of protecting against disease severity, reducing blood-stage asexual parasitemia as well as transmission, while allowing exposure to the parasite and the development of naturally-acquired immunity.

Blood-stage vaccines can be divided into those that aim to target the infected red blood cell (iRBC), and those that target the merozoite (1). Most anti-merozoite vaccines aim to elicit antibodies that bind to proteins involved in the process of red blood cell (RBC) entry thus leading to a block in RBC invasion. This antibody functionality can be routinely tested using the standardized *in vitro* assay of growth inhibitory activity (GIA) (4). A key challenge has been to identify antigens that are both well-conserved between disparate strains of *P. falciparum*, and sufficiently susceptible to antibody neutralization in the minute or so between schizont egress and RBC re-invasion (5) in a way to meaningfully impact the *in vivo* parasite multiplication rate. *P. falciparum* reticulocyte-binding protein homolog 5 (RH5) is the only protein to-date which has been demonstrated by multiple independent research groups to possess these properties (6–9). Notably, RH5 binds basigin on the surface of RBCs, forming an interaction that is essential for productive invasion of the RBC (10).

Phase I/II clinical trials are now underway to test the safety, immunogenicity and efficacy of first-generation vaccines containing the RH5 antigen (11, 12). The outcomes of these trials will serve as an important benchmark for the field of merozoite neutralizing vaccines. When interpreting the results of these clinical trials and deciding how to prioritize further research avenues, a critical question will be whether there are additional antigens (or combinations of antigens) encoded in the *P. falciparum* genome which could deliver higher efficacy than the vaccines currently being trialed that target RH5 alone.

To address this question, we generated a list of candidate antigens for a merozoite-neutralizing vaccine and tested their ability to elicit functional antibodies, comparing to RH5 as the "gold-standard" antigen. We aimed to minimize false-negatives by adopting the following approaches. First, we used protein-based vaccine immunization which, in contrast to genetic/nucleic acid-based immunization approaches such as recombinant viral vectors (7), allows for controlled administration of a fixed dose of antigen. Second, we produced recombinant protein which, wherever possible, represented the full-length ectodomain of the *P. falciparum* protein, to minimize the possibility of producing non-conformationally relevant protein fragments as initially occurred with RH5 (13–15). Third, wherever possible, rather than use Freund's adjuvant [a strong immunopotentiator that elicits extremely high titers of antibodies but which can also disrupt the fold of even the most stable proteins (16, 17)], we used AddaVax™, a squalene-based adjuvant similar in composition to the clinically-approved adjuvant MF59®.

Testing all 5,000 proteins encoded in the *P. falciparum* genome is not feasible and so in our first panel we aimed to test all of the proteins that have been the subject of promising vaccine antigen reports in the literature. We then selected a second panel, drawn from a list of 435 proteins that have been bioinformatically predicted (18) to have a role in merozoite invasion. This was 55 proteins in all. Proteins were generated for 20 of these, and then formulated in adjuvant for immunization into mice. The post-immunization mouse antibodies were harvested and then tested for activity in the GIA assay.

MATERIALS AND METHODS

Analysis of Polymorphism of Merozoite Proteins

The raw material for this polymorphism analysis was gathered from a range of sources [(19–22), Pf3K, 2012[1]]. We used sequence coverage of a gene as an initial indicator of vaccinological suitability. In a given dataset, sequence coverage is as defined by the number of Illumina reads that align to a given region of the *P. falciparum* clone 3D7 reference genome. Local drops in coverage can indicate divergence of an allele from the 3D7 reference, an A-T rich region (i.e., low complexity) or the presence of insertions/deletions. Therefore, genes with low sequence coverage, or spikes or drops in sequence coverage, were excluded from study. When counting single nucleotide polymorphisms (SNPs), we only considered non-synonymous SNPs with uniform sequence coverage and a prevalence of over 10%. This cut-off was chosen as a plausible indicator that a SNP is under some element of balancing selection. Polymorphism was assessed by counting the total number of non-synonymous SNPs along the gene, as opposed to measuring SNP density. This is because antigenic escape mutations may cluster around regions of a protein that are proximal in the tertiary, three-dimensional structure, but distant in the primary sequence. This simple counting analysis is unlikely to be confounded by the underlying base-rate SNP density across the genome, because the base rate of non-synonymous SNPs with prevalence of >10% is extremely low across the *P. falciparum* genome [(19–22), Pf3K, 2012[1]]. Genes with more than 4,000 bp (corresponding to 1,333 amino acids) were excluded due to the cost of producing synthetic genes longer than 4,000 bp.

Protein Expression and Purification

Protein sequences were uploaded to the TMHMM server (23) to predict the ectodomains of transmembrane proteins of interest (for proteins with no transmembrane region, the entire soluble protein sequence was used). Synthetized genes encoding these ectodomains (GeneArt, Germany) were cloned into a modified pENTR4 plasmid. This plasmid contains an extended version of the cytomegalovirus promoter (24) downstream of an N-terminal human tissue plasminogen activator (tPA) leader sequence and cloning site encoding "GTK" (MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGTK) and upstream of a sequence encoding a C-terminal affinity construct consisting of a biotin acceptor peptide (BAP) and either a hexa-histidine tag ("BAP-6His," final C-terminal amino acid sequence GLNDIFEAQKIEWHEHHHHHH) or a Strep tag ("BAP-Strep," final C-terminal amino acid sequence GLNDIFEAQKIEWHEWSHPQFEK) or simply a C-tag (25) with no biotin acceptor peptide, so final C-terminal amino acid sequence: EPEA. All genes were codon-optimized for *Homo sapiens*. The only exception to the above details was the "CyRPA (Gly KO)" construct (**Figure S6**), where a complete expression plasmid using a different backbone with different signal peptide and C-terminal tags was kindly gifted by the laboratory of G.

[1]Pf3K (2012). *The Pf3K Project −1.0 pilot data* release.

Wright, who had produced this plasmid [Add gene plasmid number: 50823 (26)]. Plasmids were transiently transfected into suspension HEK293E cells (250 mL typical culture volume) using a transfection method based on that described by Durocher et al. (27). Briefly, cells were grown in Expi293 Expression Medium (Thermo Fisher Scientific, USA) in a rotary shaking, 37°C and 8% CO_2, and passaged to 1×10^6 cells/mL 24 h prior to transfection. On the day of transfection, plasmid DNA was mixed with linear 25 kDa average molecular weight polyethylenimine (PEI) at a 1:2 mass ratio (Alfa Aesar, USA), and incubated for 20 min, before being added to the HEK293E cell culture. 4 days after transfection, supernatants were harvested by spinning at 500 x*g* for 10 min and supernatants clarified with a 0.22 μm filter. Proteins were purified by single-step affinity chromatography using either a HisTrap Excel Ni-Sepharose column (GE Healthcare, USA) and eluting in 400 mM imidazole; using a C-tag with elution into 2 M $MgCl_2$ (25); or dialysed extensively into phosphate-buffered saline (PBS) before application to a Streptactin Sepharose resin and elution into the supplied elution buffer (IBA, Germany). Purified proteins were exchanged into PBS using a 10 K MWCO centrifugal filter (Millipore, UK) and adjusted to 0.4 mg/mL. Proteins were resolved using 4–20% SDS-PAGE gels with reduction by 2-mercaptoethanol and visualized directly using Coomassie brilliant blue R-250 (Bio-Rad, USA), using Precision Plus protein Unstained Standards by BioRad (USA), or visualized by Western blot onto nitrocellulose membranes using streptactin-HRP conjugate (IBA, Germany) as detection reagent. PNGase treatment (NEB, USA) was performed as per manufacturer's instructions.

For the N-terminal apical asparagine-rich protein (AARP) construct in **Figure S3F**, a construct encoding I20 to D95 was cloned into an *Escherichia coli* expression plasmid with an N-terminal 6His tag followed by a tobacco etch virus cleavage site (amino-acid sequence ENLYFQGS). This construct was cloned into an *E. coli* expression host (BL21(DE3)Star, Thermo Fisher Scientific) and protein induced with 1 mM IPTG at 37°C for 4 h. Clarified soluble cell lysate was subject to Nickel-ion affinity purification (HisTrap™ Excel, GE Healthcare) following the manufacturer's instructions. This was followed by protease cleavage for removal of the 6His-tag and further size-exclusion chromatography, yielding a protein of apparent molecular weight 12 kDa.

Animal Immunization

All mouse experiments used female 6 week old BALB/c mice ($n = 4$ per group). Experiments and procedures were performed in accordance with the UK Animals (Scientific Procedures) Act Project License (PPLs 30/2889 and PA7D20B85) and were approved by the University of Oxford Local Ethical Review Body. Protein (20 μg/dose) was formulated with AddaVax™ (Invivogen, France) or Abisco®-100 (Isconova, Sweden) adjuvant, and injected intramuscularly on days 0, 21, and 42. Final serum harvest was performed on day 49. Each 100 μL dose of vaccine consisted of 50 μL protein solution at 0.4 mg/mL mixed with an equal volume of AddaVax™ or Abisco®-100 adjuvant (28), and was split across both quadriceps. For Poly (I:C), we added 50 μL of Poly(I:C) adjuvant (Invivogen, USA) at 1 mg/mL to each dose of 20 μg protein and split doses across both quadriceps. In the comparative assessment of Addavax™ and Abisco®-100, in addition to the GIA negative control groups in screening, we used chicken egg ovalbumin (OVA) (Sigma Aldrich, UK). RH5 protein, used as a functional antibody immunization control was prepared as described previously (29). When RH5 viral vectors (7) were used as a functional antibody immunization control, 10^{10} viral particles (vp) of recombinant human adenovirus serotype 5 (AdHu5) were administered intramuscularly on day 1 followed by 10^8 plaque-forming units (pfu) of recombinant modified vaccinia virus Ankara (MVA) intramuscularly on day 56, with serum harvested on day 70.

For rabbit and rat immunization, Freund's adjuvant was used to allow comparison with other studies. For S-Antigen-BAP-Strep and AARP-BAP-Strep rabbit immunization, Zika rabbits were injected intramuscularly by Biogenes (Germany). For Zika rabbits, each 400 μL dose consisted of 200 μL of protein solution at 0.5 mg/mL (100 μg dose) and 200 μL of complete Freund's adjuvant (day 1) or incomplete Freund's adjuvant (days 28 and 56). For CyRPA rabbit immunization, New Zealand White rabbits were immunized by Cambridge Research Biochemicals (UK). For these rabbits, each 400 μL dose consisted of 200 μL of protein solution at 0.5 mg/mL (100 μg dose) and 200 μL AddaVax™ adjuvant (Invivogen, France), on days 1, 28, and 56. For all rabbits the final bleed was day 63, 1 week after the final immunization. For CyRPA rat immunization, 2 Sprague-Dawley, and 2 Wistar rats were immunized intramuscularly by GenScript (Hong Kong) with 50 μg of protein formulated with complete Freund's adjuvant (day 1) and incomplete Freund's adjuvant (days 28 and 56). Rat serum was harvested on day 70 and IgG purified.

IgG Purification

Serum from immunized mice or rabbits was diluted 2:1 in protein G binding buffer (Pierce, UK) and applied to protein G sepharose columns. After washing twice with 3x serum volume of binding buffer, IgG was eluted in 10 mL protein G elution buffer (Pierce, UK) and restored to neutral pH by the addition of 300 μL Trizma pH 9.0 (Sigma Aldrich, USA). Where RBC pre-depletion was performed, 1 μg of packed 100% hematocrit RBC per 1 μg IgG was applied to sample, incubated at room temperature (RT) with agitation for 1 h. RBC were then pelleted by centrifugation at 1,000 x*g* for 5 min and the supernatant removed.

Immunofluorescence Assay

Cultures of 3D7 clone *P. falciparum,* synchronized to contain mainly late schizonts, were smeared onto glass slides to make thin films. Slides were fixed in 4% paraformaldehyde, blocked with 3% bovine serum albumin (BSA), and then probed with protein G purified serum IgG at 20 μg/mL. Secondary antibody was Alexa Fluor™ 488 goat anti-mouse IgG (H+L) at a dilution of 1:800 (Invitrogen, UK). Slides were mounted in ProLong Gold antifade reagent with DAPI (Life Technologies, USA). Images were acquired using a x100 oil-immersion objective and a cooled QICAM Fast 1,394 camera, each using the same exposure period.

Assay of Growth Inhibition Activity (GIA)

These were performed to the protocol of the GIA Reference Center, NIH, USA (4). Parasites were cultured in medium consisting of RPMI-1640 supplemented with 10% heat-inactivated human serum, 5.94 g/L HEPES, 0.05 g/L hypoxanthine, and 20 mg/L gentamycin. Synchronized parasites at 0.2–0.4% parasitemia in the trophozoite stage were grown in medium containing 20% human serum and 40 mg/L gentamycin, and then mixed with test antibody and cultured for 40 h at 1% hematocrit. Test samples were prepared by taking protein G eluate (see "IgG purification") or protein samples (see "Protein expression and purification"), and buffer exchanging three times into incomplete culture medium (lacking serum and gentamycin) using Amicon 10K MWCO centrifugal filtration devices. To deplete RBC-reactive antibodies, some samples were then incubated for 1 h with 100 μL O+ RBC at 100% hematocrit per mL of serum before centrifugation and removal of supernatant. Dialysis to exclude possibility of azide contamination was performed with Pierce MINI dialysis devices into incomplete culture medium.

Detection of parasite and inference of growth was performed using the lactate dehydrogenase method. BG98 is a rabbit anti-AMA1 purified immunoglobulin antibody pool (provided by Edmond Remarque, BPRC, Netherlands). It typically gives 80–90% GIA at 6 mg/mL and was used for quality control on assay runs.

Prediction of the GIA of an antibody mixture, GIA_{A+B}, given knowledge of the GIA of individual components (GIA_A and GIA_B), assuming Bliss Additivity between components, followed the equation (30):

$$GIA_{A+B} = [1 - \left(1 - \frac{GIA_A}{100}\right) \times \left(1 - \frac{GIA_B}{100}\right)] \times 100$$

Schizont Extract Western Blot

For **Figure S3E**, schizont and RBC extract were prepared by taking synchronized cultures of 3D7 clone schizonts or uninfected RBC, then pelleting and resuspending in a 10x volume of Laemmli buffer containing 2-mercaptoethanol, then subjecting to three cycles of freezing and thawing. Extracts were separated on 4–20% polyacrylamide gels along with Prestained Protein Marker, Broad Range (New England Biolabs, USA) and then blotted onto nitrocellulose membrane. Membranes were probed with total IgG from immunized mice at 20 μg/mL as primary antibody, then detected with alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Jackson, USA), using p-nitrophenylphosphate (pNPP) (Sigma Aldrich, USA) dissolved in water for detection.

Antigen-Specific Antibody Affinity Purification

CyRPA-specific rabbit IgG samples were generated in two stages. Total IgG was purified as above using protein G-sepharose. CyRPA-specific IgG was then purified from the total IgG using CyRPA-conjugated sepharose columns. These were generated using CyRPA (GlyKO)-C-tag protein and NHS-activated Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer's instructions. Total IgG was diluted 1:10 in 50 mM Na_2HPO_4 and applied to CyRPA-treated sepharose columns five times. After washing with 10 column volumes of 50 mM Na_2HPO_4 to remove the non-specific antibody, CyRPA-specific IgG was eluted with 0.1 M glycine and restored to neutral pH by addition of Trizma pH 9.0, before three rounds of buffer exchange into PBS using an Amicon 10K MWCO centrifugal filtration device.

ELISA

96-well Maxisorp plates were coated overnight with 100 ng/well CyRPA (GlyKO)-C-tag protein (glycan sites ablated) in PBS and then blocked with 10% milk powder. Test samples and secondary antibody (anti-mouse IgG (whole molecule) produced in goat and conjugated to alkaline phosphatase, Sigma, UK) were diluted in PBS plus 0.05% Tween-20. Alkaline phosphatase activity was measured by breakdown of pNPP using optical density at 405 nm (OD_{405}). Anti-OVA and anti-AARP titer was measured by defining the dilution factor required for a sample to yield a negative OD_{405}, defined as 0.15 using a pre-immune negative reference. For CyRPA, a standardized ELISA was also established according to principles outlined in (31) and automated by Gen5 software (BioTek, USA). Briefly, serum from the six rabbits shown in **Figures 5**, **6** was pooled to make a standard sample whose concentration of anti-CyRPA IgG antibody was defined as 8,000 arbitrary units (AU). This sample was used to construct standard curves on assay plates. Samples were diluted such that they gave an OD_{405} lying on the linear region of the standard curve (generally between OD_{405} 0.3 and 2). The AU of the starting sample was then back-calculated based on the dilution of the sample and its position on the standard curve.

Anti-CyRPA Antibody Quantification by Concentration-Free Calibration Analysis (CFCA)

Our approach to CFCA has been described previously (30). To generate a source of mono-biotinylated CyRPA for use on a streptavidin Biacore capture chip, HEK293 cell cultures were co-transfected with a plasmid encoding CyRPA (GlyKO) (**Figure S6**) and a second plasmid encoding the BirA biotin ligase, and after 4 days centrifuged and filtered to remove cell debris prior to extensive dialysis into PBS. Total IgG samples were assayed at a 1:1,000 dilution of the concentrations shown in **Figure 6C**.

Statistics

Analysis was performed using GraphPad Prism version 5.04 (GraphPad Software Inc., USA). Tests and statistics are described in Figure Legends. To determine EC_{50} values from **Figures 5E,F**, for each individual rabbit a four-parameter sigmoidal dose-response curve was fitted to the relationship between $\log_{10}$(antibody concentration) and GIA, and then used to interpolate EC_{50}s for each rabbit. To generate conversion factors for ELISA AU into μg/mL of affinity purified CyRPA-specific IgG for each individual rabbit, linear interpolation was performed between individual data points for immune rabbits and the pre-immune data point in **Figure 6A**.

RESULTS

Protein-In-AddaVax™ Immunization Induces High-Titer Antibodies in BALB/c Mice

Freund's adjuvant has been widely used in the generation of high-titer polyclonal antibodies against proteins of interest. However, its suitability for the testing of potential vaccine antigens in animal models is questionable. Evidence that it can disrupt the tertiary structure of proteins (16), along with ethical issues concerning the adverse reactions associated with its use, persuaded us to consider alternatives for an antigen screening programme in mice. A previous study in our laboratory reported AbISCO®-100 as a potent, alternative preclinical adjuvant (28) and a more refined replacement for the Freund's adjuvant system. However, the promising and on-going clinical development of Matrix-M (32) (the successor formulation of AbISCO®-100), made acquiring this adjuvant difficult at the time of performing the study and so we examined instead AddaVax™, a preclinical analog of the licensed human adjuvant MF-59 which is a squalene-based oil-in-water emulsion (33, 34). Mice immunized with chicken egg ovalbumin (OVA) formulated with AbISCO®-100 and AddaVax™ produced comparable titers of anti-OVA serum IgG antibodies (**Figure S1**) and so we proceeded with AddaVax™ adjuvant for subsequent experiments.

Production of a Panel of Protein-In-Adjuvant Pre-clinical Vaccines

Fifty-five proteins of interest were selected, falling into two categories:

The first category consisted of 15 proteins, described in the literature as having properties that are desirable in a merozoite-blocking vaccine. These proteins met at least one of the following criteria: (i) susceptibility to vaccine-induced antibody, *in vitro* or *in vivo*; (ii) established involvement in the process of merozoite egress from schizonts or invasion into new RBC; (iii) essential to parasite viability, generally defined by reported failures to disrupt the relevant gene; or (iv) strong association with protection from clinical malaria in sero-epidemiological studies (**Table S1**). Candidate antigens which have already been characterized in our laboratory such as merozoite surface protein 1 (MSP1), erythrocyte-binding antigen-175 (EBA175), RH2, and RH4 were excluded [see (7) for full list]. We have previously attempted to produce viral vectors expressing MSP2 which were not immunogenic and which were subsequently found not to express MSP2 in mammalian cells (unpublished data). We therefore also excluded this protein from the literature-selected list.

The second category contained forty proteins selected from a pool of 435 genes whose protein products are implicated bioinformatically in merozoite invasion of RBC (18). A schematic for the selection of these 40 proteins is shown in **Figure S2**. Of these 435 genes, 104 are predicted to encode a signal peptide (35). A signal peptide is likely to be an essential property for traffic to the parasite surface, potentially via invasion-associated organelles, and is also true of all validated targets of merozoite-neutralizing antibody including MSP1, apical membrane antigen 1 (AMA1), RH5, RH4, and EBA175. Eliminating 18 proteins longer than 1,333 amino acids (due to challenges in synthesizing genes more than 4,000 bp in length), and 16 proteins which have previously been tested in our laboratory as vaccine antigens left a total of 70.

To select the final 40 from this list of 70 we ranked proteins according to polymorphism, with the intention of selecting the least polymorphic (see Methods)—it should be noted that subsequent and ongoing consolidation of genomic data into the MalariaGen portal (www.malariagen.net) means that the numbers of SNPs, and especially the frequencies within the population, have changed since our accessing these data in mid-2012. However, using AMA1, RH5, and RH4 as benchmark proteins we found that polymorphism across the list of 70 was much lower than expected. AMA1, a protein affected by strain-specific neutralization, has 34 SNPs, making it substantially more polymorphic than any protein in the set of 70. However, even proteins likely unaffected by strain-specific neutralization such as RH5 (4 non-synonymous SNPs) and RH4 (3 non-synonymous SNPs) also ranked among the top third most polymorphic proteins in the set. In fact, 33 of 70 proteins were found to contain zero non-synonymous SNPs of >10% prevalence. Presumably, RH4, and RH5 are still under a minimal degree of immune pressure which is absent for the proteins with no SNPs. This result meant that our original plan to select the 40 least polymorphic proteins from the list of 70 would have resulted in the exclusion of RH5 and RH4, which was counter to our overall strategy of selecting proteins with a similar profile to RH5.

We therefore changed our strategy for triage by polymorphism, aiming to select proteins with evidence of immune pressure, but without so many polymorphisms as to risk strain-specific immunity, i.e., a panel of minimally polymorphic proteins. Excluding 30 of the proteins with no SNPs of >10% prevalence left 40 proteins that, on the level of SNPs, resembled RH5 far more than they resembled AMA1 (see **Table S2**). The final selection contained 27 proteins with 0–5 SNPs, 8 proteins with 6–15 SNPs, and 5 with 15–25 SNPs. Of the literature-selected panel of 15 proteins, 8 would also have been identified using the final bioinformatic selection algorithm (**Table S1**).

We also aimed to minimize the probability of producing small polypeptide fragments that do not fold correctly and hence fail to elicit a functional antibody response, as occurred with RH5 in early studies of this target (7, 13, 14). Therefore, where possible, we aimed to express the entire ectodomain of the 55 shortlisted proteins. An important decision when using mammalian expression systems is whether to retain or ablate predicted sites of N-linked glycosylation (defined as N-X-S/T, where X is any animo acid residue except proline). As various *Plasmodium* antigens have been shown to elicit functional anti-parasitic antibody responses without modification to the primary amino acid sequence after expression *in situ* from mammalian cells (36–38), we obtained constructs that encoded the amino acid sequence exactly as predicted from the 3D7 clone parasite reference genome for all but one antigen candidate [P113 being the exception, the synthetic gene was the same as that used in a previous study and already had N-linked glycan sequons ablated (39)]. For SEA-1 and CyRPA, for which particularly

promising vaccine antigen characteristics have been reported in the literature (40, 41), in addition to the native amino acid sequence, we also obtained constructs encoding versions of the protein with N-linked glycan sequons ablated (comparison shown in **Table S1**), in order to minimize the probability of a false-negative result in screening.

Mammalian expression plasmids encoding the 55 proteins of interest were successfully cloned and subsequently transfected into 500 mL cultures of HEK293E cells. Of the 55 different *P. falciparum* proteins, sufficient protein for a small scale mouse immunization campaign (approx. 300 μg, requiring expression at >0.6 μg/mL of cell culture supernatant) was obtained for 20 (**Figure 1**). This equates to a success rate 36.4% and is roughly in line with a previous attempt to produce a protein library using suspension HEK293 cells (42). We had significantly more success with the literature-selected panel than the bioinformatic panel (9/15 vs. 11/40, Fisher's exact test $P = 0.0329$), presumably reflecting publication bias toward proteins that can be expressed recombinantly. In a logistical regression analysis, neither protein length (OR = 0.9965, 95% CL 0.9914–1.0015) nor number of SNPs (OR = 0.8705, 95% CL 0.7125-1.0636) were predictive of expression in a statistically significant manner. Most proteins ran at a higher molecular weight than that predicted by the primary structure. Both variants of SEA-1 and CyRPA could also be expressed (**Figures 1A,B**). For SEA-1, no obvious difference in banding pattern was observed between the native version and the glycan-ablated version, and both constructs contained an additional band at approximately 75 kDa, which may represent the presence of a contaminant of SEA-1 dimer (**Figure 1A**). SEA-1 contains only two N-glycan sequons, both of which are judged unlikely to be glycosylated by the NetNGly server (http://www.cbs.dtu.dk/services/NetNGlyc/). The glycan-ablated version of CyRPA ran as a sharper band than the native version, presumably due to the presence of multiple glycoforms in the native version as CyRPA (native) contains 3 potential N-glycosylation sequons (**Figure 1B** and **Figure S6**). For many proteins, multiple smaller bands were observed, probably arising from proteolytic cleavage of the full-length construct. Given that the full-length protein was likely to be present, the presence of these smaller bands was deemed acceptable for screening purposes. Purified proteins were subsequently formulated with AddaVax™ adjuvant to produce pre-clinical protein-in-adjuvant vaccines.

Vaccines Induced Antigen-Specific IgG With Variable Neutralizing Activity

Groups of 4 mice were immunized with the vaccines over several independent experiments. Each experiment contained mice immunized with RH5 (7) as a functional antibody positive control and mice immunized with OVA as a negative control. Because production of RH5 protein at the time of vaccine preparation was not yet optimized, yield was low and therefore only the first experimental group received RH5 protein-in-adjuvant vaccine (**Figure 1A**) while subsequent groups received RH5 viral vectors. Gels in **Figure 1** show proteins immediately prior to formulation with AddaVax™ adjuvant. Following completion of the immunization phase, serum was harvested by terminal cardiac bleed. IgG was purified from the pooled sera of each group of mice and tested for binding to late schizonts by immunofluorescence (**Figure 2**). Total IgG from mice immunized with RH5 reacted strongly to schizonts whereas IgG from OVA-immunized mice did not. Of the remaining protein constructs tested, all but AARP-BAP-Strep and MSP11-BAP-Strep elicited IgG that reacted with schizonts. Although this experiment was not designed to assess localization, we did compare our results with previous studies to confirm similar staining patterns (**Table S3**). Most antibody samples showed staining consistent with previous reports, but for three proteins it was unclear whether our findings were in agreement with published work. Anti-ASP antibodies showed a punctate pattern as expected (43, 44), but additionally stained the external perimeter of the schizont. Pf92 was found to stain in a punctate manner, in contrast to a previous study using GFP-tagged Pf92 that localized to the merozoite surface (45). The GFP construct used in this study may have had non-natural trafficking, explaining the difference seen with our antibodies against native 3D7 parasite. Finally, a previous report has identified P113 as an interaction partner with RH5, and localized it to the surface of merozoites (39); however we were unable to identify any schizonts exhibiting merozoite surface staining with P113 (**Figure 2**), with staining more characteristic of the parasitophorous vacuole or iRBC surface, consistent with previous proteomics work (46).

Purified serum IgG was next screened for neutralizing activity in the *in vitro* assay of GIA at 1 mg/mL (**Figures 3A–D**). Anti-RH5 antibodies, whether raised using protein-in-adjuvant or virus vectors, had GIA ranging from 48 to 67%, whereas antibodies raised against OVA had < 5% GIA. In this initial screening, three vaccines appeared to have elicited functional antibodies which merited further study: CyRPA (**Figure 3A**), S-antigen and AARP (**Figure 3B**). All other IgG samples showed < 10% GIA at 1 mg/mL, which is indistinguishable from background GIA with anti-OVA antibodies.

Investigation of Antibodies Against AARP and S-Antigen

Given the screening results above, we next sought to further analyse the antibodies against S-antigen and AARP. Murine polyclonal IgG antibodies to AARP showed high-level GIA against both 3D7 and FVO parasites (**Figure 4A**). However, given that the murine anti-AARP antibodies showed no discernible binding to schizonts in the IFA assay (**Figure 2**), their ability to neutralize parasites in the assay of GIA raised concerns about their mechanism of action and the validity of this result. We first aimed to rule out contamination of the sample with sodium azide, which is present in small amounts in the protein G binding buffer used for IgG purification. Dialysis of the small amount of remaining antibody sample showed that small molecule contamination was unlikely (**Figure S3A**). These experiments depleted the remaining sample of IgG from the screening experiment, however a subsequent attempt to immunize rabbits with AARP-BAP-Strep did not yield functional antibodies (**Figure S3B**). Therefore, a further 10 mice were

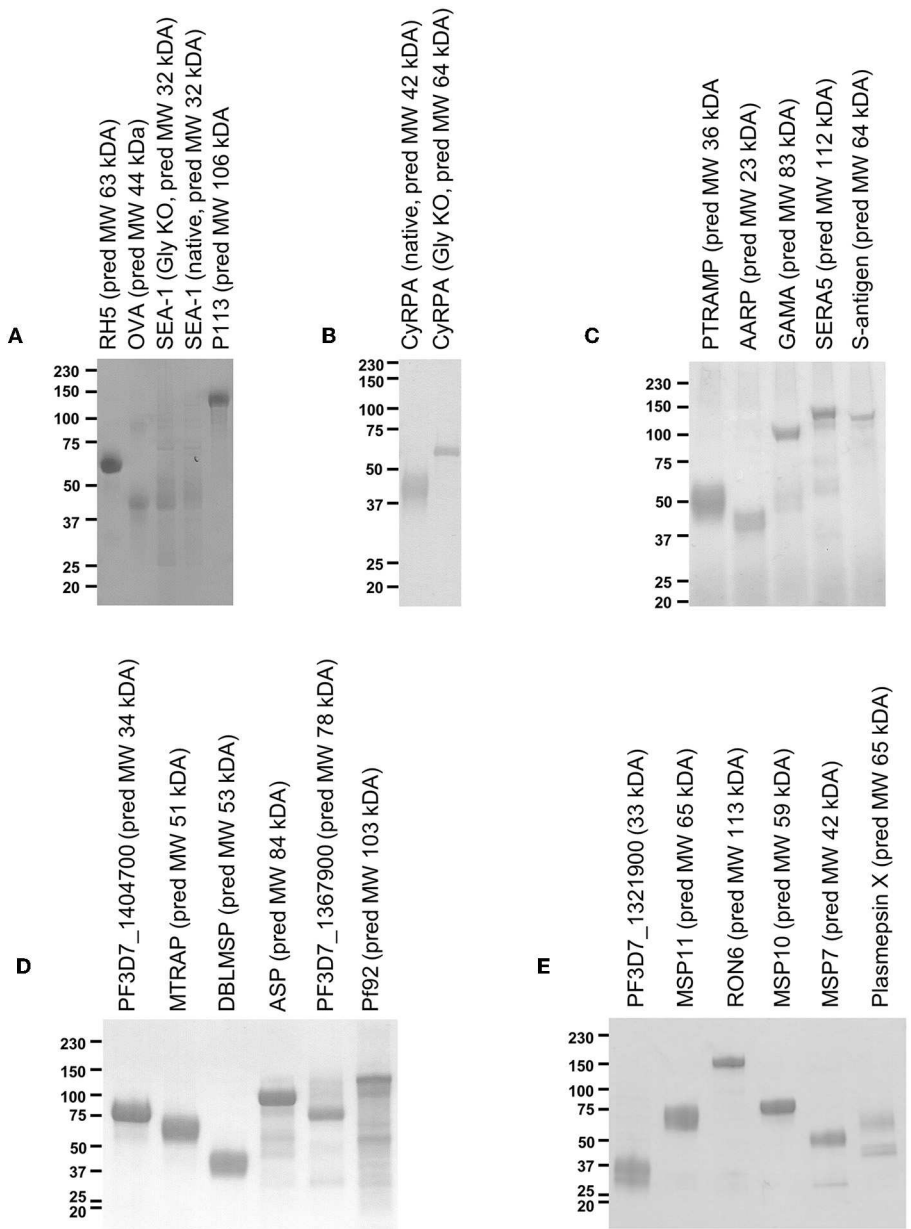

FIGURE 1 | Twenty-two protein antigens representing 20 different *P. falciparum* proteins. Just prior to formulation with AddaVax™ and immunization into mice, approximately 2 μg protein was analyzed by reducing SDS-PAGE. Four immunization campaigns were run in total, with **(A,B)** combined into one campaign, and **(C–E)** each having their own campaign. **(A)** also includes analysis of "GIA positive" and "negative" control proteins, RH5, and OVA.

immunized again exactly as for the screening study with AARP-BAP-Strep protein, and serum from pairs of mice was pooled to give five samples. Interestingly, three of the five samples showed high GIA whereas the remaining two samples showed no GIA (**Figure S3C**). To test whether the functional antibodies in the samples cross-reacted with the RBC surface, we pooled the five samples and assessed GIA after an RBC pre-incubation step (**Figure 4B**). RBC pre-treatment totally ablated all GIA of the anti-AARP antibodies in a manner comparable to the anti-basigin mAb TRA-1-85 (10) (a mAb against the RH5 receptor, used here as an anti-RBC control). Notably, these results with anti-AARP IgG contrasted with anti-AMA1 antibodies where no effect of RBC pre-incubation was seen (**Figure 4B**). These data suggested that the mechanism of GIA of IgG from mice immunized with AARP was through binding to a molecule on the RBC surface. Alternatively, the inhibitory antibodies raised by vaccination with this protein preparation may function by neutralizing the parasite, but also cross-react to a non-inhibitory epitope on RBCs. The negative anti-schizont IFA (**Figure 2**) points away from this latter conclusion.

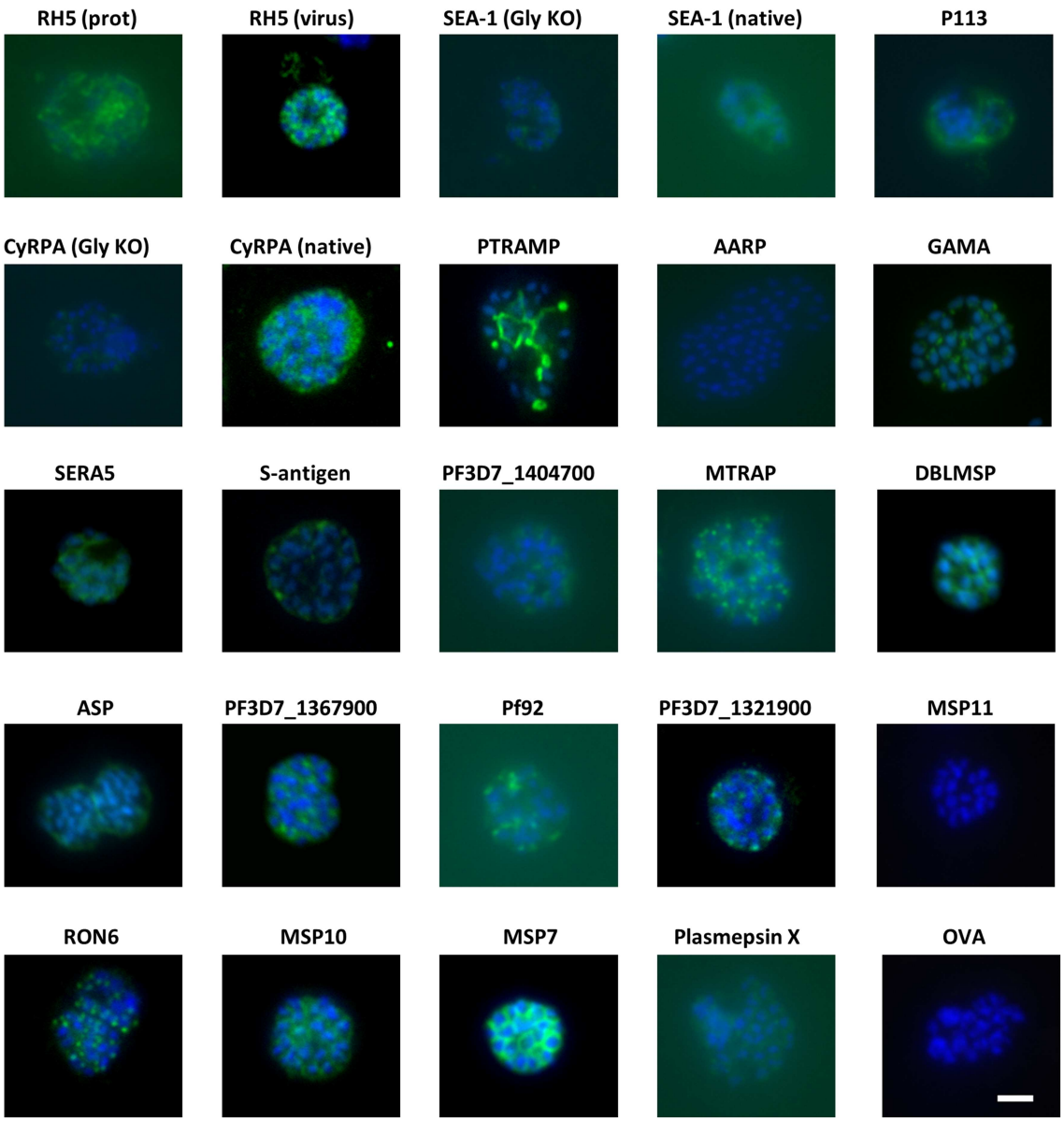

FIGURE 2 | Eighteen of twenty antigens elicit antibodies that react with *P. falciparum* schizonts. Groups of 4 mice were immunized with the proteins in adjuvants as shown in **Figure 1**. Seven days after the final injection, serum was harvested and pooled for each group before purification of IgG using protein G. The IgG was then probed for reactivity to paraformaldehyde-fixed schizonts in an immunofluorescence assay at a total IgG concentration of 20 μg/mL. All images were captured using the same microscope settings on the same day. Scale bar = 5 μm.

To further interrogate this, we prepared a new batch of AARP-BAP-Strep protein and tested its ability to reverse the GIA of the murine anti-AARP IgG antibodies. GIA from the anti-AARP antibodies could not be reversed by the addition of 2.5 mg/mL of the new batch of AARP-BAP-Strep protein, whereas 2.5 mg/mL AMA1-BAP-His protein was sufficient to fully deplete the GIA activity of anti-AMA1 IgG antibodies in the assay (**Figure 4C**). Taken together these results did not support neutralization of parasite AARP by IgG raised in our screen as a mechanism of GIA; they also suggested that antibodies against a HEK293 cell contaminant protein in the AARP vaccine were the most likely cause of the GIA. We could also not ascertain as to why this only occurred with the AARP protein vaccine in mice (noting we failed to induce growth inhibitory IgG in rabbits immunized with the same protein), and therefore we could not exclude the possibility of murine antibodies binding to a cryptic epitope such as a transient conformer of AARP or a rare glycoform, which cross-reacted with human RBC. Of note, AARP does carry two glycosylation sequons and AARP-BAP-Strep as expressed did contain PNGase-sensitive glycans (**Figure S3G**). Attempts to express AARP using a construct with glycan sequons ablated were not successful in the HEK293 system (data not shown).

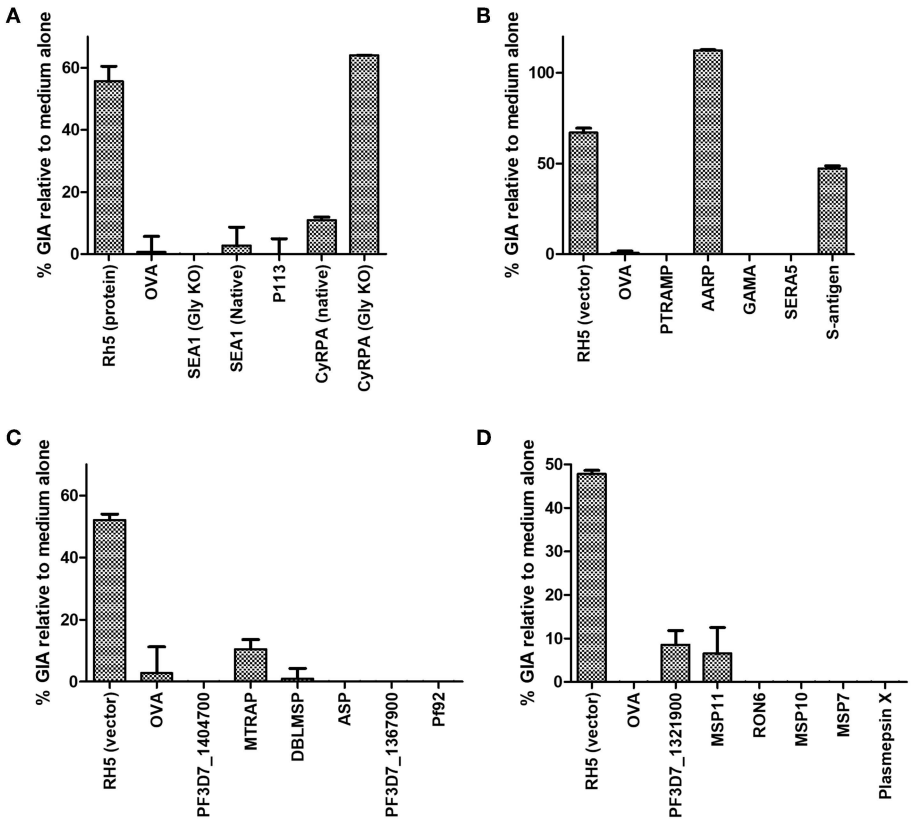

FIGURE 3 | Growth inhibitory activity of antibody specificities. The protein-G purified total IgG samples tested for schizont reactivity as shown in **Figure 2** were screened for activity in the standardized assay of GIA at 1 mg/mL. Each of panels **(A–D)** represents IgG from one independent mouse immunization campaign. Samples were not subjected to pre-incubation with RBC prior to GIA testing. Data is shown as mean of triplicate wells of a single GIA assay, and are representative of two independent assays.

Given the high level of neutralization of the antibodies, and their ability to strongly inhibit the growth of two divergent *P. falciparum* strains (**Figure 4A**) we attempted to further characterize the mechanism of neutralization by comparing the properties of the three pools shown in **Figure S3C** that did have GIA with the 2 pools that did not. However, we were hampered by the relatively small sample volume that can be obtained from mouse serum and an inability to produce functional rabbit antibodies (**Figure S3B**). The five samples shown in **Figure S3C** showed no difference in anti-immunogen ELISA (**Figure S3D**), and no consistent difference in binding to either schizont lysate or RBC lysate as measured by Western blot (**Figure S3E**). Notably, previous reports of AARP as a candidate vaccine antigen have expressed a short construct of AARP from *E. coli* [amino acids I20-D107 (9, 47)]. We therefore tested a construct mirroring this published version as closely as possible. We were only able to produce a construct encoding residues I20-D95 in *E. coli* (**Figure S3F**). We found that when formulated in AddaVax™ and immunized into mice, antibodies elicited by this construct did not have any GIA, in contrast to AMA1 (**Figure S3H**).

In light of these data, we elected to not pursue this candidate antigen any further and processed to assess antibodies against the second hit in our screen—S-antigen. Here results were more promising. Murine polyclonal IgG antibodies to S-antigen showed GIA against both 3D7 clone and FVO strain parasites (**Figure 4D**), an effect that was reproduced against the 3D7 clone using immunized rabbit polyclonal IgG antibodies pre-depleted with RBC (**Figure 4E**). Importantly, mixing of purified S-antigen protein with rabbit total IgG could reverse the GIA of S-antigen antibodies, but not RH5 antibodies (**Figure 4F**). The antibodies isolated from mice immunized with S-antigen were probed against schizont extract by Western blot (**Figure S5**) and found to react to a single band of similar size to the recombinant S-antigen in **Figure 1**. These results suggest that S-antigen can be a target of neutralizing vaccine-induced antibodies. The ability of antibodies to neutralize 3D7 and FVO parasite was surprising because S-antigen is polymorphic (**Figure S4**), with 3D7 and FVO varying substantially: even among the relatively conserved 97-amino acid N-terminus of S-antigen, there are 41 sites of amino-acid divergence, with FVO and 3D7 diverging at 27 of these (**Figure S4**).

Investigation of Antibodies Against CyRPA

In our initial antigen screen, we found a striking difference in the potency of the polyclonal IgG mouse antibodies elicited to CyRPA (Gly KO) compared to CyRPA (native), both

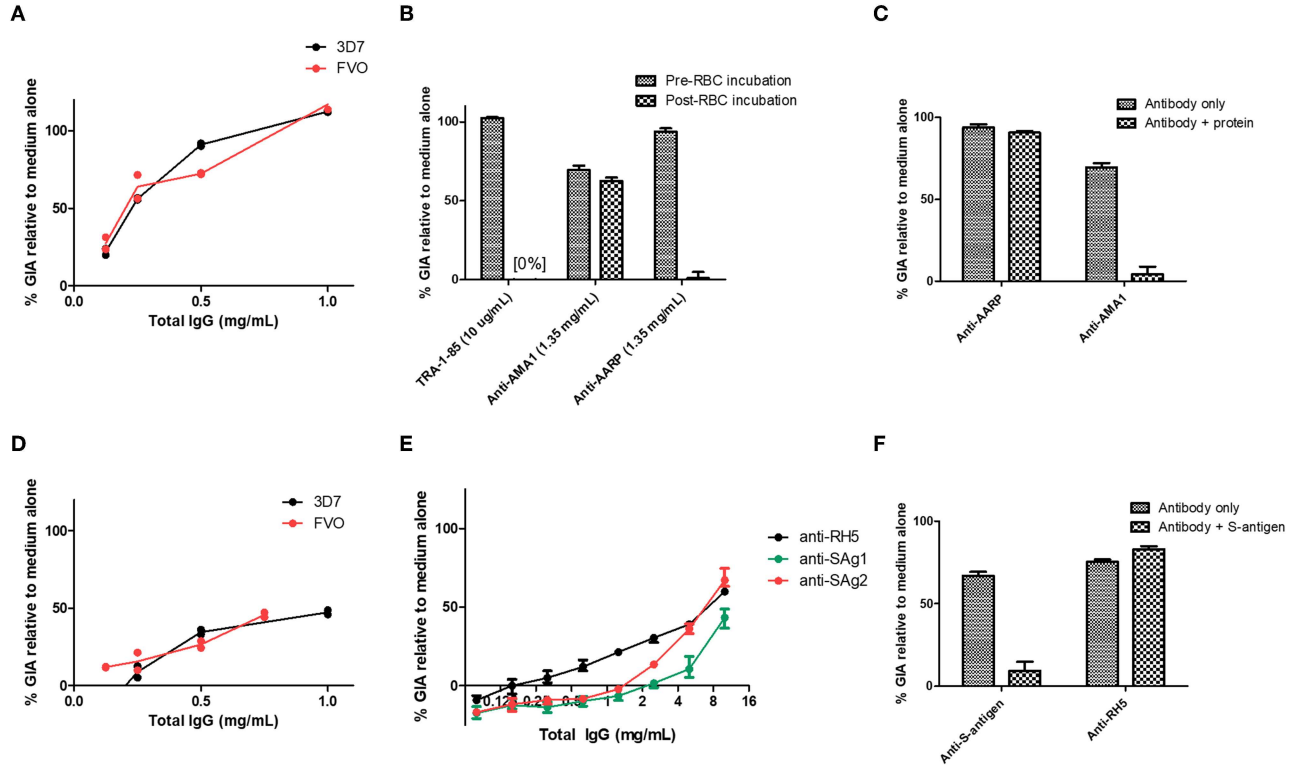

FIGURE 4 | Functional activity of anti-S-antigen and anti-AARP antibodies in the assay of GIA. **(A)** Titration of the same anti-AARP-BAP-Strep sample shown in **Figure 3B** against the 3D7 clone and FVO strain. **(B)** Effect of RBC pre-incubation on a range of mouse antibody specificities. TRA-1-85 is a mouse anti-basigin monoclonal antibody. Other samples are total IgG from immunized mice (see **Figure S3** for further details). **(C)** Pre-mixing of the relevant immunizing antigen with mouse total IgG. Final antigen concentration was 1.35 mg/mL, IgG concentration was 1 mg/mL. **(D)** Titration of the same anti-S-antigen-BAP-Strep sample shown in **Figure 3B** against the 3D7 clone and FVO strain. **(E)** Total IgG from two Zika rabbits immunized with S-antigen-BAP-Strep formulated with Freund's adjuvant system. Samples were subjected to pre-depletion with RBCs. **(A–D)** are each from a single GIA assay run due to sample volume constraints. **(A,D)**, lines are connected mean and points individual replicate wells (two per concentration). **(F)** Pre-mixing of S-antigen protein with pooled rabbit total IgG from **(E)**. Final S-antigen concentration was 1.5 mg/mL, total IgG concentration was 10 mg/mL. **(A–D)**, mean and range of four replicates. **(E,F)**, mean and range of three wells of a single GIA assay, representative of data from two replicate GIA assay runs.

quantitatively in terms of their potency (**Figure 3A**) and their ability to combine with rabbit anti-RH5 IgG antibodies (7) to give a synergistic level of GIA (**Figures 5A,B**). Consistent with a previous report on these two antigens (41), the combination of anti-CyRPA (Gly KO) and anti-RH5 IgG here showed synergy in the assay of GIA across two replicate assays. Aside from the amino acid substitutions to remove potential sites of N-linked glycosylation (S147A, T324A, T340A, schematic in **Figure S6**), these constructs differed markedly. The CyRPA (GlyKO) expression plasmid was a kind gift from Gavin Wright and colleagues, who use murine Ig signal peptide, and notably include a 20.4 kDa epitope tag on CyRPA (Gly KO) derived from rat CD4 domains 3 and 4 (CD4d3+4) (**Figure S6**) (48). However, before investigating which, if any, of these differences in construct design had led to differing qualities of anti-CyRPA IgG, we sought to reproduce the synergy result with the CyRPA (Gly KO) vaccine and immunized new cohorts of mice and rabbits with a new batch of CyRPA (Gly KO) protein. The IgG antibodies elicited by the CyRPA (Gly KO) antigen formulated with AddaVax™ reproducibly showed GIA against 3D7 clones parasites in both mice (**Figure 5C**) and rabbits (**Figure 5E**). We also tested formulation with Poly(I:C), a fully aqueous molecular adjuvant system, and saw diminished GIA activity by comparison to AddaVax™ adjuvant in mice (**Figure 5D**). The rabbit anti-CyRPA antibodies also showed high-level neutralization of the FVO strain (**Figure 5F**). However, the synergy observed in our initial experiment (**Figure 5A**) was not observed in subsequent experiments using IgG purified from newly immunized mice (**Figures 5C,D**) or rabbits (**Figure 5G**); only an additive effect was observed.

Given these conflicting data, we sought to confirm the validity of the initial result showing synergy between antibodies against CyRPA (Gly KO) and RH5. It was observed in the initial experiment (**Figure 5A**) that the anti-CyRPA (Gly KO) purified IgG antibodies caused agglutination of the RBC in the GIA assay, and we sought to exclude this as a mechanism of GIA. We found that pre-depletion of the mouse IgG antibody sample with human RBC ablated the agglutination observed and had no effect on the GIA, either as a single specificity or its ability to synergize with anti-RH5 IgG (**Figure S7A**). To exclude any

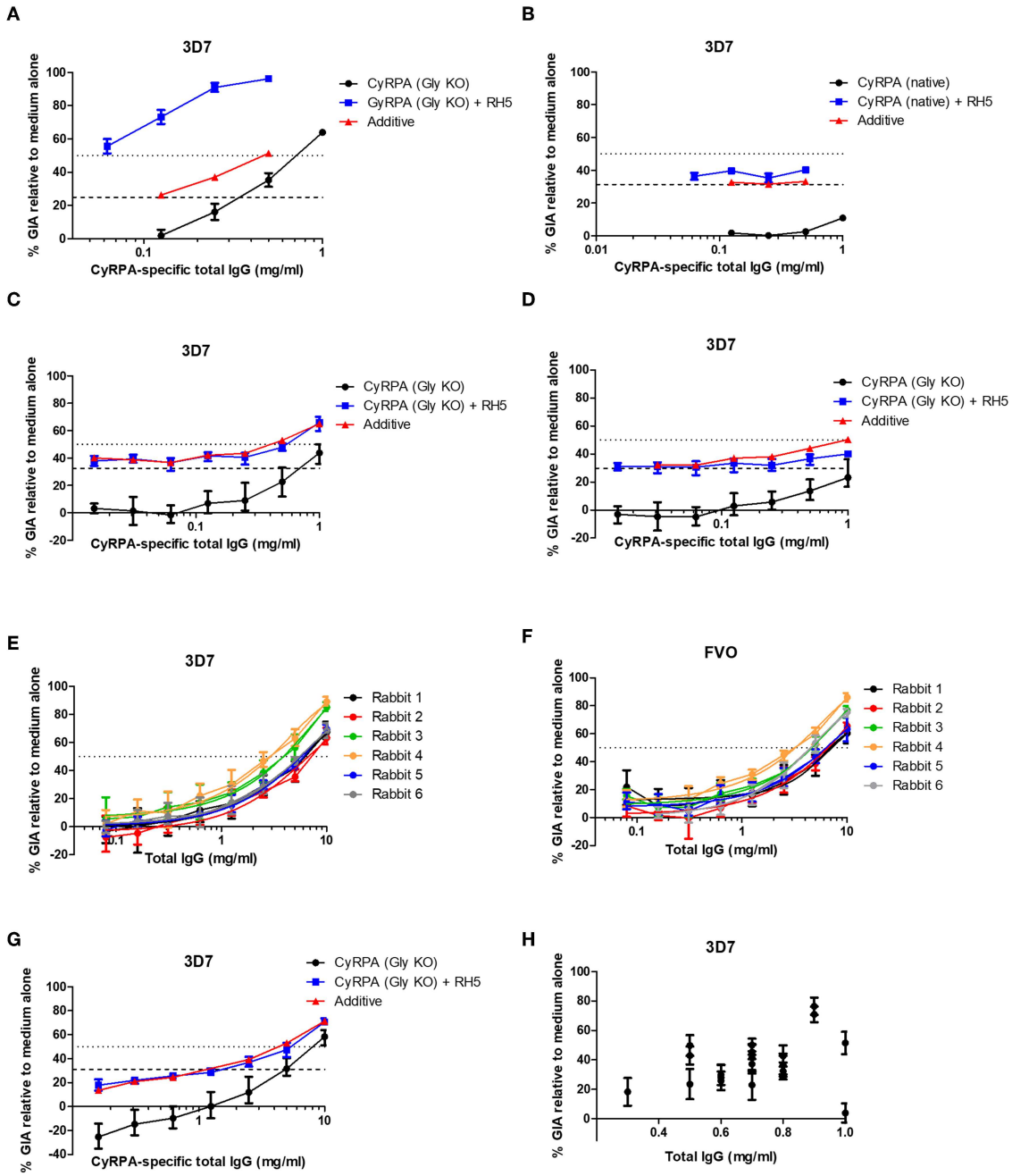

FIGURE 5 | Functional activity of anti-CyRPA antibodies in the *in vitro* assay of GIA. **(A,B)** Titration of the anti-CyRPA purified IgG samples (from **Figure 3A**) against 3D7 clone parasites, with (blue line) and without (black line) addition of a fixed concentration (1.25 mg/mL) rabbit anti-RH5 purified IgG antibodies. Dashed line shows the activity of the anti-RH5 IgG alone tested at the single fixed concentration and red line shows the calculated activity of the mixture, assuming Bliss additivity (30). Dotted line indicates 50% GIA. **(C)** A new sample of mouse anti-CyRPA (GlyKO) IgG was generated and assessed as in **(A)**. **(D)** A new sample of mouse anti-CyRPA (Gly KO) IgG was generated using an identical immunogen to **(A,C)**, but using Poly(I:C) adjuvant for formulation. **(E,F)** Six rabbits were immunized with CyRPA (Gly KO) in AddaVax™ and their total IgG tested in a titration series for GIA against 3D7 clone and FVO strain parasites. **(G)** Pooled IgG antibodies from the six rabbits in panels **(E,F)** were tested for synergy against the 3D7 clone with anti-RH5 IgG antibodies as in **(A,B)**. **(H)** GIA of total IgG purified from 20 individual mice immunized with CyRPA (Gly KO) and AddaVax™, each tested at a different concentration as defined by IgG yield in the sample. All panels show representative results from two replicate GIA assays, except **(E)** which was repeated 3 times. Each point shows the mean of three replicate wells; error bars show the range of three replicates wells.

effect of possible anti-rat CD4d3+4 antibodies we also generated recombinant CyRPA with a C-terminal four amino acid C-tag (25), which contained the same CyRPA (Gly KO) sequence but lacked the CD4d3+4 domain, called CyRPA (Gly KO)-C-tag (see **Figure S6** for construct comparisons). A vaccine containing CyRPA (GlyKO)-C-tag yielded functional antibodies comparable

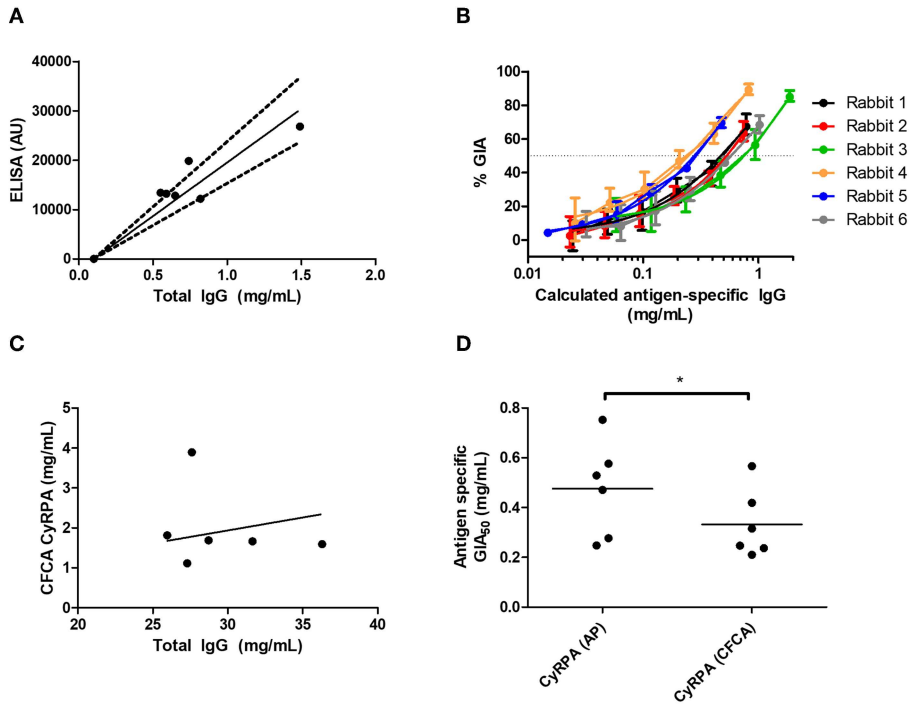

FIGURE 6 | Potency of vaccine-induced anti-CyRPA antibodies from six individual rabbits. See **Figure S8** for experimental schematic. A second batch of IgG was purified from the six rabbits shown in **Figures 5E,F**, as well as one pool of pre-immune IgG, using protein G. **(A,B)** Calculation of antigen-specific IgG concentration by affinity purification (AP) and ELISA analysis. **(A)** Total IgG from six individual rabbits was passed over a CyRPA-sepharose column and the six eluates were analyzed for total protein concentration by OD_{280}, and assayed for CyRPA binding by ELISA, to generate a conversion factor for each rabbit. Points are individual rabbits, solid line is mean conversion factor (shown for information only—not used in calculations), dashed lines 95% CI (actual calculations were performed with separate conversion factors for each individual rabbit). **(B)** GIA data from **Figure 5E** transformed using the conversion factors determined for individual rabbits in panel A, to show CyRPA antigen-specific IgG concentration. Points are means of 3 assay well replicates, error bars show range of three replicates. **(C)** Calibration-free concentration analysis (CFCA). Total IgG was analyzed for total protein concentration by OD_{280}, and anti-CyRPA antigen-specfic IgG concentration by CFCA. Pre-immune IgG gave a sub-detectable CFCA response. **(D)** Interpolated GIA EC_{50}s for the six CyRPA immunized rabbits. Points are individual rabbits, bars are means.

to the parental CyRPA (Gly KO) construct as a single specificity, however, these also showed additivity, but not synergy, with anti-RH5 IgG antibodies (**Figure S7B**), meaning that a role for anti-CD4 IgG cannot be excluded in the results from **Figure 5A**. Consequently, in a final effort to understand the mechanism of the synergy seen in **Figure 5A**, we immunized with CyRPA (Gly KO) and harvested IgG from individual mice, aiming to isolate monoclonal antibodies (mAbs) from mice whose serum polyclonal IgG showed synergy with anti-RH5 antibodies. IgG yields from individual mice were variable, necessitating GIA screening at a range of concentrations (**Figure 5H**). Although IgG from most mice showed GIA, not one of the IgG samples isolated from these 20 mice showed synergy with anti-RH5 antibodies and isolation of mAbs was not pursued (data not shown). Therefore, given the inconsistency of synergy observed in our study, we attempted to replicate as closely as possible the initial report of anti-CyRPA and anti-RH5 IgG antibody synergy, which used rat immunization with Freund's adjuvant (41). A pair of Wistar rats and a pair of Sprague Dawley rats were immunized with CyRPA (Gly KO) in Freund's adjuvant. Total IgG isolated from these rats showed 60–90% GIA at the highest concentrations tested, and some degree of additivity with anti-RH5 rabbit IgG, but no synergy was seen (**Figures S7C–F**). Thus, these data suggest that anti-CyRPA IgG raised in mice, rats and rabbits can elicit substantial GIA and that this is most consistently additive when combined with anti-RH5 rabbit IgG.

Assessment of Anti-CyRPA Specific IgG Potency

Given immunization against CyRPA could reliably elicit functional antibodies, we next aimed to better assess CyRPA's suitability as a candidate vaccine antigen by quantifying its susceptibility to vaccine-induced polyclonal antibody. We and others have previously reported similar analyses for polyclonal antigen-specific IgG against RH5, AMA1, and MSP1 in different species ranging from mice through humans (1, 4, 30). Here, for rabbit anti-CyRPA antibody, the mean total IgG concentration giving 50% GIA (EC_{50}) against the 3D7 clone parasite was 5.07 mg/mL (95% CI, 3.61–6.54, **Figure 5E**) and against the FVO strain was 5.34 mg/mL (95% CI, 3.83–6.86, **Figure 5F**). However, the antigen-specific IgG is typically only a small fraction of the total IgG; so we next determined the proportion of total IgG which is CyRPA-specific using two methods: (i) affinity purification of specific IgG using chromatography columns loaded with CyRPA antigen; and (ii) surface plasmon resonance analysis by calibration-free concentration analysis

(CFCA) using recombinant CyRPA coated onto a capture chip. The experimental schema is shown in **Figure S8**.

When using the affinity-purification method, CyRPA-specific IgG was purified from total rabbit IgG using CyRPA-immobilized sepharose columns. The total protein concentration (assumed to be all antigen-specific IgG) was then analyzed by OD_{280} measurement as well as measured for anti-CyRPA responses in arbitrary units (AU) by ELISA (**Figure 6A**). This was used to calculate a conversion factor for ELISA AU to a specific concentration of anti-CyRPA rabbit IgG antibodies in mg/mL for each individual rabbit (for information only, mean conversion factor: CyRPA-specifc IgG concentration (μg/mL) = 0.046 (ELISA AU)−0.1; slope 95% CI 0.058–0.043). We then measured the ELISA units of the IgG stocks used to perform the GIA in **Figure 5E** and replotted GIA against CyRPA-specific IgG (**Figure 6B**).

For CFCA, total IgG from individual rabbits was analyzed to assess the concentration of anti-CyRPA antibodies in samples whose total protein concentration was measured by OD_{280} (**Figure 6C**). For the pre-immune rabbit IgG pool, the anti-CyRPA CFCA response was sub-detectable. For the six immune IgG samples, the mean proportion of CyRPA-specific IgG as measured by CFCA was 6.78% of the total IgG (95% CI, 2.86–10.7%). With the proportion of total IgG that was specific for CyRPA now determined for each rabbit, GIA was replotted against CyRPA-specific IgG (not shown), and the antigen-specific GIA EC_{50} values calculated with each method were compared (**Figure 6D**). The CyRPA-specific rabbit IgG GIA EC_{50} was calculated to be 470 μg/mL by the affinity purification method (95% CI: 270–680 μg/mL) and 330 μg/mL for the CFCA method (95% CI: 190–480 μg/mL), with the figure determined by affinity purification significantly higher than by CFCA ($P = 0.031$, Wilcoxon matched-pairs signed rank test). Both figures suggest that relatively high Ag-specific antibody concentrations are required for CyRPA polyclonal IgG mediated GIA.

DISCUSSION

RH5 has emerged as a leading blood-stage vaccine candidate antigen, and vaccination with this antigen appears to elicit a potent growth-inhibitory antigen-specific antibody response (1, 12). A recombinant protein vaccine called RH5.1 based on this antigen is currently in Phase I/II clinical trials (11). The objective of this study was to shed light on whether there are other antigens that could supersede or synergize with RH5 in their ability to elicit growth-inhibitory antibodies against blood-stage *P. falciparum*. This will inform the approach taken to improve on the present RH5 vaccine and/or develop next-generation vaccine formulations targeting multiple antigens to achieve even higher levels of growth inhibitory antibodies.

To meet this objective, we tested a range of merozoite proteins in as unbiased a manner as possible, testing their ability to elicit antibodies with functional GIA. RH5 was used as a "gold-standard" antigen. Fifty-five proteins of interest were selected for study: 15 were selected from the literature as having properties desirable in a vaccine antigen; while the other 40 proteins were selected on the basis of a bioinformatically predicted role in merozoite invasion (18). These 40 proteins were selected from a shortlist of 70 on the basis of having a higher number of SNPs. Of the 55 proteins selected, 20 were successfully expressed, and for 18 we were able to raise an antibody response that showed binding to fixed schizonts. While an expression success rate of 20/55 proteins is high compared to an expression library effort using a bacterial system (49), and in line with a recent study using suspension HEK293 cells (42), this leaves 35 proteins unaddressed by this study. Alternative expression systems using insect cells (29) and protozoan cells (50) may achieve a higher success rate in future studies.

When tested for function in the *in vitro* assay of GIA, three antibody specificities showed functional activity when tested alone: S-antigen; AARP and CyRPA. While our finding that S-antigen can be a target of neutralizing antibody is novel, AARP and CyRPA have been the subject of previous publications highlighting promise for these proteins as clinical candidate vaccine antigens. We therefore scrutinized the latter two antigens particularly closely, to help inform the immediate prioritization of antigens for inclusion in the next generation of clinical vaccine candidates. For S-antigen, purified IgG samples from immunized mice and rabbits (that had also been pre-depleted with RBC) were able to inhibit growth in a reproducible manner, and this effect could be reversed by pre-incubation with S-antigen protein. S-antigen has been largely unstudied since the 1980s, and was included in this screen because of statements in review articles authored by Cowman *et al.* that S-antigen is refractory to genetic ablation (51, 52). Interestingly, while most of the 14 other proteins that were initially selected based on literature reports as showing a role in merozoite invasion would also have been identified using the bioinformatics approach used to identify the other 40 proteins (18), S-antigen was not identified bioinformatically as having a role in this process. It is therefore surprising that S-antigen appears to be the target of neutralizing antibody in the GIA assay. Given the extreme variability of S-antigen between strains [**Figure S4**, and also (53)], the ability of antibodies raised to the 3D7 clone sequence of S-antigen to neutralize the FVO strain was particularly unexpected. Although we did not follow-up this result further, our data suggest S-antigen may warrant re-visiting in future vaccine studies, with particular emphasis on defining the neutralizing epitopes and understanding the effect of polymorphism between strains.

Different antibody samples raised against AARP did not show consistent functional GIA, and further investigation suggested the function was likely mediated through off-target binding of antibodies to the RBC. Notably, previous publications ascribing a high level of neutralization activity to anti-AARP antibodies do not describe an RBC pre-incubation step in the purification and preparation of IgG from serum (9, 47), and we were unable to reproduce these results with a similar, albeit not identical, immunogen. Our negative results for AARP in rabbits are consistent with those described in a separate, recent antigen screen (54). Although we elected to not study this antigen any further, future identification of the RBC moiety bound by the neutralizing anti-AARP IgG samples could potentially yield

important information about the mechanism of invasion of RBC by merozoites.

In the case of our final "hit," immunization with CyRPA-based constructs could reproducibly elicit antibodies from mice, rats and rabbits with invasion-blocking activity. The first mouse IgG sample we produced exhibited synergy when combined with anti-RH5 rabbit IgG antibodies across numerous replicate assays, in line with an earlier report on this antigen combination (41). However, the fact that multiple subsequent immunizations did not yield GIA with this synergistic property, including rats immunized using an almost identical protocol to that used by Reddy et al. (41), suggests that it is highly likely a result of rare antibody clones binding to specific epitopes. It could be that use of AddaVax™ adjuvant biased the antibody response toward a non-synergistic repertoire of epitopes, although a similar result was obtained when using Freund's adjuvant in rats. Although synergy between RH5 and CyRPA IgG specificities was inconsistent, the combination did consistently result in additive GIA in our hands, in agreement with some other reports. Bustamante et al. found that the combination of RH5 and CyRPA total IgG from immunized rabbits alternated between synergy and sub-additivity in the GIA assay depending on the concentration used (54), while Favuzza *et al.* appear to observe an additive interaction between RH5 and CyRPA mAbs (55). Given CyRPA, RH5 and Ripr form a complex (56, 57), it is possible that individual antibody clones that block or stabilize different aspects of complex formation could mediate either synergistic or additive interactions with anti-RH5 antibodies. However, the relationship between antibody potency and the role of the target antigen protein in merozoite invasion is far from clear: we have previously found that antibodies to RH5 interact synergistically with antibodies to RH2, RH4, and EBA175, additively with antibodies to MSP1 and sub-additively with antibodies to AMA1 (30), none of which are known to interact directly with RH5.

Further characterization of the rabbit anti-CyRPA IgG indicated that the antigen-specific GIA EC_{50} is substantially higher than that of vaccine-induced antibodies to RH5 (30) and AMA1 (58), and more akin to the levels required when targeting MSP1 (4). These data would argue against prioritization of CyRPA over RH5 for inclusion in a single-subunit blood-stage malaria vaccine. However our results are inconsistent with recent work published by Bustamante et al., who found that anti-CyRPA rabbit antibodies are at least as potent as anti-RH5 antibodies (54). Indeed, the GIA EC_{50} reported by Bustamante *et al.* for total IgG from CyRPA-immunized rabbits against 3D7 clone parasites is 0.3834 mg/mL. This is more than 10 times lower than our total IgG GIA EC_{50} of 5.07 mg/mL, and equivalent to our calculated antigen-specific GIA EC_{50}. Both studies used very similar immunogens and also the same contractor for rabbit immunization (Cambridge Research Biochemicals). The differences in the GIA results could have arisen from other methodological factors, which could include GIA assay format and potentially differences in adjuvant selection and/or immunization regimen. We, and others, have noted quite marked differences in rabbit immunization studies with regard to overall endpoint titres raised against protein antigens, which may easily explain the discrepancy of the results for total IgG. Moreover, it is wholly possible the immunization protocols affected the quality of anti-CyRPA IgG induced in the rabbits, and this would suggest careful monitoring of the protein formulation and anti-CyRPA IgG quality is warranted in future studies. Nevertheless, the results in our present study are concordant with those published by Reddy et al. (41) and in line with the observation that the most potent anti-CyRPA mAbs yet described have a GIA EC_{50} of at least 250 μg/mL (55, 59–61); by contrast, the most potent anti-RH5 mAbs have a GIA EC_{50} of ∼15 μg/mL (62). Taken together, it does appear that total IgG from RH5-based immunization tends to be more potent than IgG from CyRPA-based immunization. However, if the epitopes responsible for synergy between anti-RH5 and anti-CyRPA IgG could be identified and antibodies robustly elicited against them in combination, this could form the basis for a more effective bivalent next-generation blood-stage vaccine.

A final unexpected result from our screen was the failure of antibodies to both the glycan-ablated or the native version of SEA-1 to exhibit any GIA. Raj et al. found that SEA-1 antibodies are associated with protection from clinical malaria, and also showed that vaccination with PbSEA-1 can protect mice from *P. berghei* rodent malaria (40). Our results do not necessarily contradict these findings, because protection from malaria by anti-SEA-1 antibodies might well be mediated by Fc-dependent immune effector functions not measured in the GIA assay such as complement fixation and myeloid cell activation. However, our finding that anti-SEA-1 antibodies did not have GIA does conflict with Raj et al.'s finding that anti-SEA-1 serum antibodies, as well as mAbs, can block growth in *in vitro* cultures (40). It is possible that our protein was not folded correctly, or was insufficiently immunogenic when immunized into mice with AddaVax™ as an adjuvant, leading to a negative result. Fortunately Raj *et al.* produced mAbs to SEA-1 (40), and testing these in the standardized assay of GIA as operated by the International GIA Reference Laboratory should resolve this discrepancy.

With respect to the process used to select the "bioinformatically selected panel" of 40 proteins, even in retrospect it is difficult to say whether the approach taken was optimal. Of the 15 literature-selected proteins, 8 would have been identified with the bioinformatic selection algorithm. Among the 2 robust hits from the screen, CyRPA would have been selected using the bioinformatic approach whereas S-antigen would not have (**Table S1**). This result does suggest that there may be other invasion-blocking candidate antigens which were not identified as part of the "Merozoite invasion sub-network" by Hu et al. (18). It is difficult to say whether our decision to prioritize the moderately polymorphic proteins at the expense of non-polymorphic proteins was the right one. CyRPA and S-antigens, the two robust hits from this study, both came from the literature-selected panel. CyRPA has one SNP of >10% prevalence, which would mean that had it been in the 'bioinformatically-selected' panel it would have been brought forward whether we selected the 40 most polymorphic or the 40 least prevalent genes. S-antigen is so polymorphic that with the data available at the time it was not possible to actually count SNPs, and even with data available now a full discussion of polymorphism in S-antigen is beyond the scope

of this paper. Both of these antigens elicit antibodies capable of neutralizing 3D7 and FVO, which differ markedley in their S-antigen sequences, suggesting that polymorphism is not always predictive of strain-specific neutralization.

While this study has focused on the assay of GIA, as one defined mechanistic correlate that is causative of protection against blood-stage *P. falciparum* (63) it should be recognized that alternative assays of anti-merozoite antibody efficacy do exist, including phagocytosis assays (64), the assay of antibody-dependent respiratory burst (65), and the assay of antibody-dependent cellular inhibition (66). Antibody performance in each of these assays has been shown to correlate with protection from clinical malaria in the context of naturally-acquired humoral immunity. However, there are technical challenges with using these assays for antibodies derived from different species as in this study. We selected the assay of GIA because our goal was to benchmark against antibodies to RH5, which are functional in this assay.

In summary, no antibody specificity in this study outperformed anti-RH5 antibodies in the GIA assay. Neither did any polyclonal antibody specificity robustly synergize with or enhance the GIA of anti-RH5 antibodies, although additivity with anti-CyRPA IgG was generally consistent. While we attempted to make this screen as comprehensive as possible, we were not able to express every candidate antigen in HEK293 cells, and new antigens continue to be described that were not included in our original list of proteins. Bustamante *et al.* have very recently reported that SERA9, RAMA, MSRP5, and EBA181 are targets of neutralizing antibody which potentially synergize with anti-RH5 antibodies (54). The RH5-interacting protein (Ripr) is another protein with a strong rationale for testing as a vaccine antigen, but which has not been well studied due to the difficulty of expressing it in bacterial and mammalian cell systems (26, 67). It now remains a priority to test these proteins in human compatible vaccine-delivery systems to find out if their inclusion in a multi-valent vaccine offers a realistic possibility of improving on RH5 alone.

ETHICS STATEMENT

Animal experiments and procedures were performed in accordance with the UK Animals (Scientific Procedures) Act Project License (PPLs 30/2889 and PA7D20B85) and were approved by the University of Oxford Local Ethical Review Body.

AUTHOR CONTRIBUTIONS

JI: wrote the paper. JI and SD: conceived the study. JI, DA, MS, KW, AD, GL, JC, DP, and MH: produced and characterized proteins. JI, LB, DP, and SdC: performed animal immunization studies. JI, RB, JM, and DQ: performed GIA assays. SS and HB: performed affinity purification and Biacore analysis on CyRPA-specific antibodies. JI and JW: selected genes of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01254/full#supplementary-material

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Conflict of Interest Statement: JI, DA, AD, KW, SC, MH, and SD are named inventors on patent applications relating to RH5 and/or other malaria vaccines, antibodies and/or immunization regimens.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Induction of Plasmodium-Specific Immune Responses Using Liposome-Based Vaccines

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In the development of vaccines, the ability to initiate both innate and subsequent adaptive immune responses need to be considered. Live attenuated vaccines achieve this naturally, while inactivated and sub-unit vaccines generally require additional help provided through delivery systems and/or adjuvants. Liposomes present an attractive adjuvant/delivery system for antigens. Here, we review the key aspects of immunity against *Plasmodium* parasites, liposome design considerations and their current application in the development of a malaria vaccine.

Keywords: malaria, *Plasmodium*, immunity, adjuvant, liposomes, vaccine

INTRODUCTION

Malaria vaccine development has been a focus of research since the 1940s when inoculation with homologous inactivated sporozoites and/or serum resulted in control of parasitemia amongst immunized domestic fowls (1, 2). Follow-up studies also showed that monkeys and ducks were protected against *Plasmodium (P) knowlesi* and *P. lophurae* following vaccination with killed, adjuvanted parasites (3, 4). Additionally, in 1967, it was demonstrated for the first time, that immunization with irradiated sporozoites protected mice against *P. berghei* (5). Despite these early promising findings, an effective vaccine for malaria still eludes scientists with only one vaccine candidate, RTS, S, receiving a positive scientific opinion from European regulators and it is currently approved for use in pilot implementation trials in 3–5 epidemiologically distinct locations in sub-Saharan Africa (6, 7). The complexity of *Plasmodium* parasites, antigenic polymorphism and failure to maintain long-lived immune responses calls for continued efforts in the search for novel vaccines which can effectively prevent *P. falciparum* and *P. vivax* infections (8).

A major bottleneck in the development of vaccines against infectious diseases is the failure to initiate robust innate immune responses and subsequent potentiation and maintenance of downstream adaptive immune responses. This is achieved naturally with live attenuated vaccines while inactivated and sub-unit vaccines require delivery systems and/or adjuvants for efficient presentation to the immune system and additional stimulation to enhance potency (9). To address this, careful selection of adjuvants and delivery systems needs to be considered early in the vaccine development process. However, only a handful of adjuvants have been licensed or tested for use in human vaccines and these are summarized in **Table 1**.

TABLE 1 | Clinically tested and licensed vaccine adjuvants (10, 11).

Adjuvant	Status of vaccine adjuvant
Aluminum-based salts (Alum)	Licensed for tetanus, diphtheria, pneumococcus vaccines (12).
Mf59 (an oil-in-water emulsion consisting of squalene, Tween 80 and span 85)	Licensed for influenza vaccines (13, 14).
AS01: Liposome-based adjuvant comprised of Monophosphoryl Lipid A (MPLA) and QS-21	Tested in phase III malaria and shingles vaccine trials (15, 16).
AS02: squalene emulsion comprised of MPLA and QS-21	Tested in phase II malaria trials (17).
AS03: An oil-in-water emulsion comprised of squalene, Tween 90 and α-tocopherol	Licensed for influenza vaccines (18, 19).
AS04: comprised of aluminum hydroxide and MPLA	Licensed for Cervarix vaccine against HPV and Fendrix against hepatitis B (20–22).
ISA-51 Montanide: Mineral oil with a Mannide monooleate emulsifier	Licensed for Cimavax vaccine against non-small cell lung cancer (NSCLC) (23).
Virosomes: Comprised of influenza virus envelopes reconstituted in liposomes	Licensed for hepatitis A (Epaxal) (22, 24) and Influenza vaccines (Invivac, Inflexal) (25).
CAF01: Cationic liposomes comprised of dimethyldioctadecylammonium bromide (DDAB) and trehalose 6,6-dibehenate (TDB)	Tested in phase I HIV and tuberculosis vaccine trials (26, 27).
IC31: TLR9 agonist	Tested in phase I tuberculosis vaccine trials (28–30).
Poly I:C: TLR3 agonist comprised of repeating units of double stranded inosine and cytosine	Tested in phase I/II cancer vaccine trials (31).
Imiquimod: TLR7/8 agonist	Tested in phase II therapeutic vaccine trials against vulval intraepithelial neoplasia (32)
SE/SE-GLA: Squalene emulsion co-formulated with TLR4 agonist GLA	Tested in phase I influenza vaccine trials (33–36)
ISCOMS, ISCOMATRIX/ Matrix-M^{TM}: Lipid-based adjuvants formulated with cholesterol and saponins	Tested in a phase I vaccine trials against HCV, HPV and influenza (33–35, 37) (NCT02905019)
Recombinant CTB: B subunit of cholera toxin	Licensed for the cholera vaccine, Dukoral (38)

First proposed by Gregoriadis and Allison in 1974 as immunological adjuvants (39), liposomes are a promising vaccine adjuvant/antigen delivery system. Historically well-known as drug carriers, liposomes are self-assembling phospholipid vesicles capable of incorporating and protecting antigens from degradation, as well as facilitating antigen delivery to professional antigen presenting cells (APCs) (9, 40–43). Liposomes generally act by depot formation resulting in enhanced uptake by APCs and subsequent induction of the desired immune responses. To date, the extensive use of liposomes can be attributed to their safety profile, biocompatibility, biodegradability, versatility, and plasticity and therefore they present an attractive platform for malaria vaccine development.

In a natural malaria infection, the acquisition of clinical immunity is slow, spanning several years of repeated exposure, and it is not sterile (44). An ideal vaccine capable of inducing sterile immunity against the different life-cycle stages of malaria will need to induce a qualitatively and/or quantitatively different immune response to that induced during natural infection immunity (45). Since stage-specific immunity to malaria requires humoral and cell-mediated immune responses, the ideal vaccine-induced responses should preferably be comprised of both forms of responses. However, for rational vaccine development, a clear understanding of the complex nature of immunity to malaria is required and this in turn may help to inform the selection of an appropriate adjuvant/delivery system. This review highlights key aspects of the immune response to malaria, design considerations of liposomes, and their current application in malaria vaccine development.

IMMUNITY TO MALARIA

Plasmodium parasites, the causative agents of malaria, are obligate intracellular organisms which undergo a complex life-cycle in the vertebrate host broadly divided into: the mosquito stage which occurs in the vector; the pre-erythrocytic stage which occurs in the vertebrate host's liver; and the erythrocytic stage which occurs in the blood of the vertebrate host (46). At all life-cycle stages, the immune responses induced following infection differ significantly and a clear understanding of these responses will inform the vaccine development process.

Pre-erythrocytic Stage Immunity to Malaria

The pre-erythrocytic stage of malaria infection is clinically quiescent, probably due to the low number of sporozoites inoculated by the mosquito while taking a blood meal. During this stage, studies in mice have shown that antibodies can control infection through immobilization of sporozoites by inhibiting sporozoite motility and subsequent invasion of hepatocytes (47, 48). Following natural infection, studies demonstrated the existence of pre-erythrocytic antigen-specific antibodies to *P. falciparum*; however, their role remains unclear (49–51). To date, the best model that has enabled the study of pre-erythrocytic immune response mechanisms has utilized irradiated sporozoites in both humans and animals. Radiation-attenuated sporozoites retain the capacity to infect hepatocytes but cannot develop into an erythrocytic infection. Studies in rodent models involving inoculation of radiation-attenuated sporozoites demonstrated that antibodies were involved in the enhanced clearance of sporozoites, reduction in sporozoite motility and inhibition

of hepatocyte invasion (52, 53). In clinical studies, induction of antibodies to the circumsporozoite protein (CSP) following immunization with the *P. falciparum* sporozoite (PfSPZ) vaccine has been shown to partially correlate with protection (54–58). Furthermore, following immunization with RTS, S/AS01, high CSP-specific antibody titers are induced and are a surrogate measure of protective efficacy for this vaccine candidate (16, 59–63).

Cell-mediated immunity following inoculation of radiation-attenuated sporozoites has also been shown to contribute to vaccine-induced sterilizing immunity to *Plasmodium* infection in both mice and humans (54–58, 64–72). Studies in rodent models, however, indicate that a network of cellular mechanisms mediates immunity to pre-erythrocytic infection (64–66, 68, 70–72). Initial studies in mice immunized with irradiated *P. yoelii* 17XNL sporozoites reported that cytotoxic $CD8^+$ T cells mediated protection against a wild-type challenge of infectious sporozoites (64, 65). Further studies indicated that IL-12 from liver antigen presenting cells (APCs) stimulated $CD8^+$ T cells and Natural killer (NK) cells to produce interferon (IFN)-γ. IFN-γ, in turn, induces the infected hepatocytes to produce nitric oxide (NO), which subsequently kills the parasite in the hepatocyte (66, 68, 71). In addition, the balance between IL-2, IL-10, IL-12, and IFN-γ results in an inflammatory response that contributes to pre-erythrocytic immunity (70, 72). In human volunteers, both $CD4^+$ and $CD8^+$ T cell responses against pre-erythrocytic antigens have been observed following immunization with irradiated sporozoites (54–58, 67, 69).

Controlled infection immunization (CII) studies, involving experimental sporozoite inoculation via infectious mosquito bites with concurrent chemoprophylaxis provides another model for investigating pre-erythrocytic immunity to malaria. These studies have shown that a protective polyfunctional T cell response to pre-erythrocytic antigens predominantly characterized by the production of IFN-γ, tumor necrosis factor (TNF), and IL-2 is induced following CII (73–76). Purified IgG against *P. falciparum* sporozoites, obtained from CII trial participants was also shown to inhibit liver stage infection in a humanized liver-chimeric mouse model (73, 75, 77).

Erythrocytic Stage Immunity to Malaria

During the erythrocytic stage, clinical signs, and symptoms of malaria manifest as the parasites invade and replicate in RBCs. Studies in animal models have demonstrated that the innate immune system is involved in the initial recognition of blood-stage parasites, promotion of inflammation, inhibition of parasite growth and potentiation of the adaptive immune response (78, 79). *Plasmodium falciparum* pathogen-associated molecular patterns (PAMPs) such as glycosylphosphatidylinositol (GPI) anchors (Toll-like receptor 2 [TLR2]), hemozoin (NOD-like receptor containing pyrin domain 3 [NLRP3] inflammasome), CpG-containing DNA motifs bound to hemozoin (TLR9) and AT-rich DNA motifs (unidentified cytosolic receptor) have been shown to trigger an inflammatory cascade by binding pattern recognition receptors (PRRs) on the surface of innate immune cells (79–83). This interaction of PAMPs and PRRs results in the production of pro-inflammatory cytokines (IL-12 [p70], IFN-γ and TNF) by APCs, as well as regulatory cytokines (IL-10 and TGF-β) that have been implicated in immunity and pathogenesis to blood-stage malaria infection (79, 84, 85).

Several innate immune cells such as dendritic cells (DCs), macrophages, mast cells, neutrophils, NK cells, natural killer T (NKT) cells, and γδ T cells have been implicated in this initial immune response (85–88). *Plasmodium* parasites have been shown to modulate DC maturation and function resulting in the induction of regulatory T cells which in turn modulates $CD4^+$ T cell responses, suppressing protective immune responses while averting immune-mediated pathology (85). IL-12 production by DCs has also been implicated in the production of IFN-γ by NK cells and $CD4^+$ Th1 cells resulting in the control of parasite growth (85). Understanding the balance between protective and immunopathologic responses following DC activation and maturation might have favorable implications in designing vaccines to prevent severe malaria (85). *In vitro*, NK cells have been shown to be an early source of IFN-γ, promoting the destruction of infected red blood cells by activated macrophages (79, 89). γδ T cells and monocytes on the other hand have been associated with elevated levels of TNF, IL- 10, IP-10, IL-6, macrophage inflammatory protein (MIP)-1β and MIP-1α which is linked with severe disease (90). Furthermore, downregulation of γδ T cell responses following repeated exposures to *Plasmodia* has also been implicated with better tolerance to clinical malaria (91, 92). Given their specificity for restricted TCR ligands, γδ T cells present an attractive target for a vaccine to protect against severe disease (45, 90).

Antibodies play a role in naturally acquired immunity to erythrocytic stage malaria, as it has been shown that the passive transfer of immunoglobulin from immune donors resulted in the reduction of parasitaemia and clinical disease among semi-immune recipients from East Africa as well as non-immune Thai patients (93–95). Antibodies may function by inhibiting merozoite invasion of RBCs (96), binding to pRBCs and enhancing clearance by the spleen (97, 98), as well as opsonizing pRBCs, resulting in phagocytosis by macrophages (99, 100).

Cell-mediated immunity against the erythrocytic stage is primarily mediated by $CD4^+$ T cells, as demonstrated in both murine and human models. Studies showed that mice depleted of $CD4^+$ T cells developed very high parasitaemia and were unable to control the infection compared to mice depleted of $CD8^+$ T cells, which developed mild parasitaemia that subsequently resolved; this indicated a clear role of $CD4^+$ T cells in erythrocytic stage immunity (101, 102). Additionally, adoptive transfer of $CD4^+$ T cells was shown to confer protection and control parasitaemia in immunodeficient mice (103). Further investigation of the role of $CD4^+$ T cells demonstrated that during the acute phase of infection, there was a significant upregulation of an IFN-γ-specific $CD4^+$ T cell (Th1) response followed by an IL-4-specific $CD4^+$ T cell-mediated (Th2) antibody response during the chronic phase (104). These data indicate that early activation of Th1 cells enables control of the infection via effector mechanisms such as macrophages, followed by a Th2 response which activates B cells to clear the parasite in the later stages of the infection in mice (105, 106).

In human studies, the role of $CD4^+$ T cells was demonstrated when volunteers were infected with low doses of blood-stage *P. falciparum* followed by drug cure (107). In this study, volunteers appeared to be protected against a homologous challenge infection with immunity associated with an IFN-γ-specific $CD4^+$ T cell response and nitric oxide synthase (NOS) production in the absence of detectable antibodies (107). However, a follow-up study suggested that residual drug may have contributed to the apparent protection (108). Another study showed that stimulation of T cells obtained from children living in Papua New Guinea resulted in parasite-specific IFN-γ and TNF responses, which were associated with protection against clinical episodes of malaria (109). More recently, studies in African children showed that $CD4^+$ T cells may play an important modulatory role in the development of blood-stage immunity (110, 111). Higher IFN-γ/IL-10+ specific-$CD4^+$ T cell responses were observed amongst children heavily exposed to malaria compared to children with low exposure indicating that $CD4^+$ T cells may play an important immunomodulatory role in the pathogenesis of childhood malaria (110). Additionally, the induction of IL-10-producing $CD4^+$ T cells amongst highly exposed children may interfere with the development of immunity, which may have implications for vaccine development (111).

T follicular helper cells (Tfh) are a subset of $CD4^+$ T cells, capable of providing B cell help as well as activating follicular B-cell responses (112–114). Recent studies in mice showed that Tfh cells play a critical role in controlling *P. chabaudi* blood-stage infection via activation of IL-21 mediated responses (115). Therefore, since humoral responses are critical to the erythrocytic stages of *Plasmodium*, an in-depth understanding of the activation and maintenance of Tfh cells during malaria will be critical in designing blood-stage vaccines (115).

Regulatory T cells (Tregs) are another $CD4^+$ T cell subset implicated in the maintenance of immune homeostasis and control of excessive pathogen-driven inflammatory responses (116, 117). Following *Plasmodium* infection, uncontrolled production of pro-inflammatory cytokines is associated with pathology in both mice and humans (118–121) and anti-inflammatory cytokines (TGF-β and IL-10) are known to be critical in the modulation of this inflammatory response (118, 120, 122, 123). The immunomodulatory function of IL-10 and TGF-β is associated with Tregs whose role in rodent malaria remains unclear. Some studies have shown that Tregs are critical in the control of pro-inflammatory responses associated with pathology (124, 125) while other studies have associated upregulation of Tregs with detrimental outcomes (124, 126, 127). These discrepancies may have been dependent on the rodent parasite strains utilized in the study (128). Tregs may inhibit protective immune responses resulting in enhanced parasite growth if induced early in infection but may also limit immune-mediated pathology (45, 128–130). Clinical studies have shown that acute infection with *Plasmodium* parasites resulted in upregulation of Tregs which positively correlated with augmented parasite load and subsequent disease severity amongst these individuals (131–134). Given this background, a vaccine capable of inducing Tregs with the ability to protect against the immunopathology associated with malaria infection would be desirable if parasite persistence is required for the maintenance of protective immune responses. However, since Tregs are known to inhibit protective immune responses, treatment with antimalaria drugs at the time of vaccination may be necessary to "normalize" the pre-existing immune response and ensure induction of the appropriate vaccine-specific responses (45).

The role of $CD8^+$ T cells in defense against blood-stage parasites remains unclear due to the fact that mature RBCs do not express MHC class I molecules. However, *in vitro* studies have shown that both *P. falciparum* and *P. vivax* are able to invade erythroblasts—immature erythrocytes that possess a nucleus and express MHC class I molecules (135, 136). Additionally, studies in mice demonstrated that blood-stage parasite antigens were cross-presented by CD8-α^+ DCs, inducing parasite-specific $CD8^+$ T cell responses capable of lysing APCs (137). These findings indicated the possible relevance of $CD8^+$ T cells in erythrocytic immunity to *Plasmodium*. Indeed, additional studies in mice demonstrated that parasitized erythroblasts activated $CD8^+$ T cells in an antigen-specific manner (138). This contact-dependent Fas—Fas Ligand (FasL) interaction of $CD8^+$ T cells with the parasitized erythroblasts results in the exposure of phosphatidylserine (PS) on the erythroblast surface (139). Cells displaying PS on their surface are rapidly phagocytosed by macrophages. Thus, $CD8^+$ T cells in conjunction with macrophages are able to mediate immunity to a blood-stage malaria infection in mice (139). Earlier studies in a *P. yoelii* infection model demonstrated that professional APCs might cross-present parasite-derived peptides on MHC class I to $CD8^+$ T cells leading to cytotoxicity through the production of IFN-γ, perforin and granzyme B (140). Furthermore, parasite-specific $CD8^+$ T cells have also been shown to clear infected reticulocytes, which express MHC class I molecules, via the secretion of IFN-γ and expression of granzyme B (141).

These data highlighted in the studies above provide an insight into the complex nature of immune responses elicited following infection with malaria parasites. Understanding the balance between protective immunity and immunopathology is critical for the development of an ideal vaccine capable of inducing both humoral and cell-mediated immune responses against different life-cycle stages of the malaria parasite. To achieve this balance, careful selection of antigen delivery systems and adjuvants during the vaccine development process is paramount and the pliability of liposome-based platforms can be utilized for this purpose.

DESIGN CONSIDERATIONS OF LIPOSOMAL VACCINE FORMULATIONS

Based on their design, liposome vaccine formulations can be tailored to achieve desired immune responses and adjuvant properties by modification of vesicle physicochemical factors (summarized in **Figure 1**), such as lipid composition, charge, PEGylation, antigen encapsulation, and addition of immunomodulators (9, 40–43).

The choice of phospholipid has been shown to enhance the adjuvanticity of liposomes. Long chain lipids tend to form

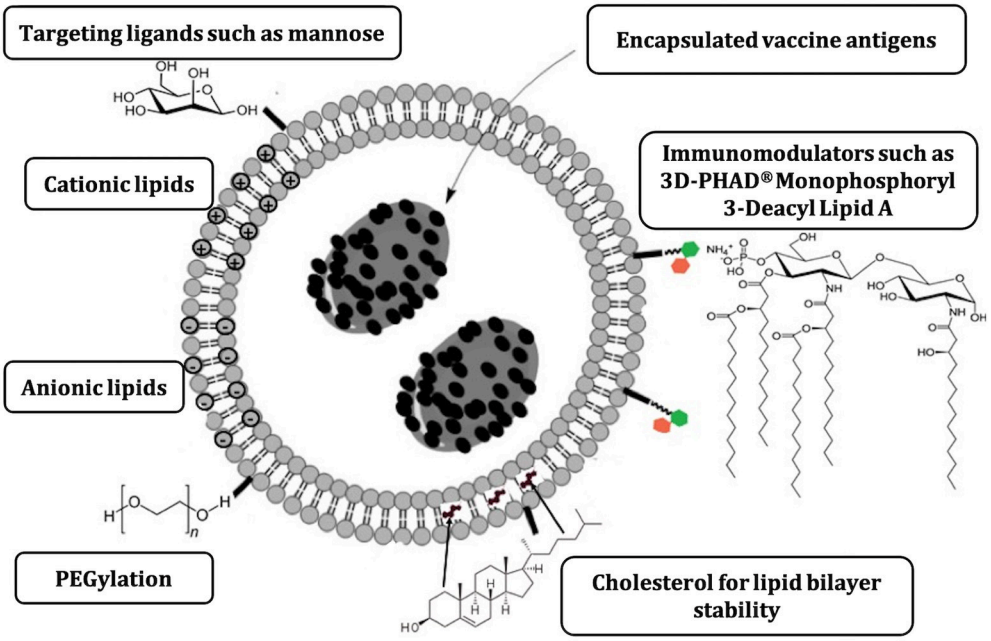

FIGURE 1 | Major liposome physicochemical characteristics that can be modified to direct immune responses elicited following vaccination (9).

rigid ordered bilayer structures while those with shorter tails tend to form fluid and disordered vesicles (9). Immunization of animals with liposomes formulated with long chain lipids such as dimethyldioctadecylammonium bromide (DDAB) and distearyol derivative of L-α-phosphatidyl choline (DSPC) resulted in stronger antigen-specific antibody responses when compared to animals that received liposomes formulated with shorter chain lipids (142). Additionally, the stability of the lipid bilayer can be enhanced further with the addition of cholesterol within the liposome formulation resulting in improved antibody responses compared to formulations without cholesterol (143–145).

Positively charged (cationic) liposomes formulated with saturated acyl chain lipids (with a quaternary ammonium head group) have been shown to promote the binding of antigen at the site of injection stimulating interaction with APCs to elicit a robust Th1 cytokine response (146). In contrast, highly fluid unsaturated acyl chain liposomes that are rapidly cleared from the injection site result in lower activation of APCs as measured by the decreased expression of DC co-stimulatory molecules CD40 and CD86 (146). Additionally, cationic liposomes have been shown to promote retention of higher levels of antigen at the injection site, resulting in a depot-effect allowing continuous attraction of APCs and subsequent induction of robust cell-mediated immune responses comprised of IFN-γ, IL-2, TNF, and IL-17 (147). On the other hand, negatively charged (anionic) and neutral liposomes were rapidly cleared from the injection site resulting in lower activation of APCs and lower Th1 as well as Th17 cytokine responses (9, 147, 148).

The inclusion of polyethylene glycol (PEG), known as PEGylation, has been extensively used for the stabilization of liposomes (149). PEGylation has been shown to mask the charge, due to the hydrophilic chains of PEG extending out from the surface of the liposomes subsequently reducing the electrostatic retention of antigen to the surface of these vesicles (150, 151). Additionally, PEGylation influences lipid packaging reducing the number of bilayers and resulting in reduced vesicle size (151). This modification of liposome size and antigen adsorption properties by PEG was shown to reduce depot formation, resulting in reduced IgG2b antibody and Th1 (IFN-γ) cytokine responses as well as an elevated Th2 (IL-5) cytokine response compared to non- PEGylated liposome formulations (150, 151).

The particle size of liposomes has also been shown to impact adjuvanticity and direct the development of the resulting cell-mediated immune response (42). Studies have shown that the immune response induced following administration of small-sized liposome vesicles (10–100 nm) was skewed toward Th2 whilst larger vesicles (400–2,500 nm) induced a Th1 response characterized by augmented IFN-γ and IgG2a production (152). The differences in the profiles of the induced immune response of large vs. small vesicles could be due to differences in antigen processing and trafficking to lymph nodes. Large-sized vesicles (560 nm) were shown to be more efficiently phagocytosed and processed by macrophages compared to smaller vesicles (155 nm) (153). Additionally, trafficking of liposome particles to lymph nodes has been shown to be size dependent, with small-sized vesicles (20–200 nm) freely draining to and specifically targeting lymph node-resident cells, while large-sized vesicles (500–2,000 nm) require dendritic cells for trafficking from the injection site to lymph nodes (154). More recently, immunization using a formulation containing large-sized cationic liposomes (∼500 nm) resulted in enhanced splenocyte proliferative responses and reduced IL-10 production compared

to small sized liposomes (∼100 nm) (155). Interestingly, smaller unilamellar liposomes (70 nm) were reported to stimulate higher IgG titers than larger unilamellar (400 nm) but not large submicron size multilamellar liposomes in mice (156). The potency of multilamellar liposomes can be explained by more efficient antigen protection against degradation in their multiple lipid bilayers.

Liposomes can be modified to incorporate additional lipophilic immunomodulators within or attached to the lipid bilayer to enhance adjuvanticity. Such immunomodulators are crucial in the activation of the cells of the innate immune system via PRRs which recognize PAMPs on the surface of pathogens, subsequently activating the adaptive immune system. The activation of innate immune cells such as dendritic cells and macrophages requires the use of Toll-like receptor (TLR) and NOD-like receptor (NLR) type PRRs to direct a robust immune response (157). Therefore, the use of synthetic PRR agonists has been predicted to be critical in the formulation of liposome-based vaccine adjuvants (9, 158).

Liposome formulations can be customized by incorporating PRR agonists that mediate activation and maturation of APCs which in turn facilitates the uptake and processing of liposome-associated antigens resulting in potent cell-mediated immune responses (9). The most widely used PRR agonist monophosphoryl lipid A (MPLA), a TLR-4 agonist, has been used in licensed vaccine formulations Fendrix (hepatitis B) (159) and Cervarix (human papillomavirus) (160). A synthetic analog of MPLA, 3′-O-desacyl-4′-monophosphoryl lipid A formulated with *Quillaja saponaria* Molina, fraction 21 (QS-21) saponin is included in the liposome-based GSK Adjuvant System 01 (AS01) and has been tested in human studies for the malaria vaccine RTS, S (Mosquirix) (16), as well as a shingles sub-unit vaccine HZ/su which demonstrated over 90% efficacy amongst elderly persons (15). Similarly, liposomes can be tagged with sugars such as mannose to target them to lectin-like molecules on APCs to facilitate phagocytic uptake thereby promoting MHC class II involvement and, via cross presentation, MHC class I. This targeting of liposomes to different uptake pathways may aid in directing the resulting immune response toward a mixed Th1/Th2 response (9, 161).

The route of administration of particulate antigen delivery systems such as liposomes has been shown to affect the type and magnitude of immune response induced. Interestingly liposomes can be even administered orally; however, they need to be extensively modified to improve their stability in the gastrointestinal tract and their mucosa adhesive properties (162). In a cross-sectional study in mice, the intramuscular, intradermal and intralymphatic routes of administration were associated with intermediate to high induction of IgG2a and IFN-γ cytokine production while the subcutaneous route was associated with low elicitation of IgG2a and IFN-γ cytokine production (163). These data indicate that the route of administration is critical in the generation of the desired immune response and should be considered while interpreting immunological data following immunization with liposome-based vaccine formulations. Together, it is evident that the versatility and plasticity of liposomes facilitates the tailoring of the desired immune responses, as well as enhanced adjuvanticity and this can be exploited for the development of a malaria vaccine.

UTILITY OF LIPOSOMES IN MALARIA VACCINE DEVELOPMENT

Liposomes are increasingly becoming used in a number of malaria vaccine candidates targeting the different life-cycle stages. The use of liposomes in the development of sporozoite-stage malaria vaccines dates back to the mid-1980s where tetrapeptide antigens (asparagine-alanine-asparagine-proline) derived from the repetitive region of the circumsporozoite (CS) protein of *P. falciparum* sporozoites were conjugated to carrier proteins and were incorporated into liposomes. These studies demonstrated that liposomes containing carrier protein-conjugated peptide induced a potent humoral immune response which was further enhanced when lipid A was incorporated in the liposome formulation (164–169). The aforementioned studies laid the foundation for the development of RTS, S, the only vaccine against malaria that has received approval for use in pilot implementation trials in sub-Saharan Africa (7). The RTS, S vaccine construct is made of the central repeat region (amino acids 210-398) (R) and the C-terminal region containing the T-cell epitopes of CSP (T), fused to hepatitis B surface antigen (HBsAg) (S), co-expressed in *Saccharomyces cerevisiae* yeast and self-assembled with unfused HBsAg antigen (170, 171). These hybrid virus-like particles (VLPs) were co-formulated with GSK's proprietary liposome-based adjuvant system, AS01, which contains potent immunostimulants, MPLA and QS-21 that was selected over AS02, an oil-in-water emulsion adjuvant following evidence of enhanced antigen-specific antibody and CD4+ T-cell responses as well as improved efficacy in large-scale clinical studies (61, 172–175). The level of and mechanism of immunity induced by RTS, S in endemic settings are topics of much research. Over 4 years of follow up, the level of protection ranged from 18 to 36%, depending on the age of the child and whether the child received 3 or 4 doses of vaccine. Protection was clearly greater in the early months after vaccination, but waned rapidly after that and there was a 'negative efficacy' during the 5th year in some children (176). This is a sub-optimal response and as such the mechanism of immunity came under great scrutiny. Evidence suggests that the level of antibody to the CS protein and serum levels of IFN-γ post vaccination both correlate with protection (177, 178). A major concern, has been the rapid diminution of antibody levels over time, in the face of parasite exposure. The problem is not that RTS,S is not immunogenic. The adjuvant system is one of the most potent there is for human use and is used elsewhere with great effect. It has made Shingrix a highly successful vaccine where even in the elderly there is 90% efficacy. Also, the problem is not with boosting *per se*, as each dose of RTS,S is accompanied by a rapid rise in antibody titer. In our view, the main problem is that even though RTS,S has a powerful adjuvant system, it is still not powerful enough. Titres wane after vaccination and natural infection will not boost. The main

reasons for this appear to be: (i) antigenic polymorphism; and (ii) that the dose of sporozoites that individuals are exposed to (and which express the CS protein) are simply too low to boost or maintain antibody levels. T cell epitopes present on the CS protein which are incorporated into the vaccine are polymorphic (179), and this polymorphism does contribute to the low efficacy (180). The T-cell epitopes are known to be non-cross-reactive (181). An additional factor may relate to liposome design *per se*. Indeed, efforts are currently underway to remodel RTS, S such that each HBsAg particle expresses CSP thereby increasing the concentration of parasite antigens (182). Here, CSP-HBsAg fusion proteins were co-expressed in *Pichia pastoris* yeast which in the presence of a tightly regulated inducible alcohol oxidase (AOX1) promoter allows production of a higher density of hybrid VLPs (182, 183). This vaccine construct, co-formulated with Matrix-M ™, a saponin-based liposomal adjuvant is now undergoing clinical testing NCT02905019[1].

In the development of transmission-blocking vaccines, gel core liposomes encapsulating Pfs25, an antigen expressed on zygotes and ookinetes of *P. falciparum* and a leading transmission-blocking vaccine (TBV) candidate, have been tested in mouse models (184). Gel core liposomes are a stabilized form of liposomes bearing a core of biocompatible polymer inside the lipid vesicle which serves to prevent the rapid release of antigen content from liposomes (184, 185). Following 2 intramuscular injections with gel core liposomes, Pfs25-specific antibody responses were observed in immunized mice, and these were maintained for up to 8 weeks. Additionally, strong Th1 cytokine (IL-2 and IFN-γ) responses were elicited and these responses were augmented when the gel core liposomes were formulated with CpG oligodeoxynucleotide (CpG-ODN) (184, 185).

A cationic adjuvant liposome formulation (CAF01) consisting of DDAB, synthetic mycobacterial cordfactor as an immunomodulator, and merozoite surface protein 1 (MSP1) antigen derived from *P. yoelii* genomic DNA, *Py*MSP1, has been tested in pre-clinical studies as a blood-stage malaria vaccine (186). Compared to the Alum adjuvanted vaccine formulation, immunization with CAF01- *Py*-MSP-1 resulted in significantly higher antibody and IFN-γ cytokine responses. Furthermore, following challenge, immunization with CAF01- *Py*MSP1 resulted in significant control of parasite growth (186).

Tyagi et al. (187), utilized a liposome-mediated transdermal immunization approach to deliver *P. falciparum* merozoite surface protein-1 (PfMSP-1) antigens through intact skin to antigen presenting cells in the skin (187). Similar to observations following immunization with CAF01- *Py*MSP1(186), durable and stronger parasite-specific humoral responses were observed up to 10 weeks post-immunization. Additionally, robust cell-mediated responses critical in immunity against blood-stage malaria parasites were induced following transdermal administration of elastic liposomes loaded with PfMSP-1 antigens when compared to Alum based vaccine formulations (187). Collectively, these data underscore the substantial superiority of liposome-based formulations over aluminum-based vaccine adjuvant formulations in the induction of parasite-specific immune responses to malaria.

More recently, our group formulated liposomes with mannosylated lipid core peptides (MLCPs) as targeting ligands for the delivery of whole blood-stage parasite antigens to professional antigen presenting cells (188). Immunization with these mannosylated liposome formulations resulted in the induction of significant $CD8^+$ T cell responses; immunized mice demonstrated better control of parasitemia as well as extended survival following challenge, when compared with control mice, availing an alternative delivery system for inactivated whole parasite antigens (188). Together, the studies above indicate that liposomes are being considerably used in malaria vaccine development for targeting all of the different life-cycle stages and their pliability can be further explored to develop a multi-stage vaccine.

CONCLUDING REMARKS

The quest for a vaccine against malaria continues despite the partial success with RTS, S/AS01, which showed modest efficacy in phase III clinical trials (16). Given the complex network of immune responses elicited following infection with *Plasmodium* parasites, an ideal vaccine should aim to induce the appropriate life-cycle stage-specific-antibody and cell-mediated responses capable of protecting against disease and immunopathology. The versatility of liposomes can be exploited to achieve an optimal formulation via the use of charged lipids to promote antigen retention at the injection site (depot-effect) (186), inclusion of targeting ligands to promote uptake by professional APCs (188), to control their stability, release of antigen, enhance antigen protection, and include immunomodulators (40, 43). Additionally, as the poor efficacy of most malaria vaccines evaluated thus far in field trials has been attributed to antigenic polymorphism, the use of whole parasite antigens (188) in liposome formulations needs to be explored further in malaria vaccine development. In summary, the modification of vesicle physicochemical properties may be further exploited to design an optimal liposome formulation with a high level of efficacy required for complete eradication of malaria by 2030 (8).

AUTHOR CONTRIBUTIONS

AS, DS, and MG drafted and reviewed original manuscript. AG, MZ, MS, and IT critically reviewed original manuscript and provided important intellectual content.

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A Synthetic Nanoparticle Based Vaccine Approach Targeting MSP4/5 Is Immunogenic and Induces Moderate Protection Against Murine Blood-Stage Malaria

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Malaria remains a significant health problem in many tropical and sub-tropical regions. The development of vaccines against the clinically active blood-stage of infection needs to consider variability and polymorphism in target antigens, and an adjuvant system able to induce broad spectrum immunity comprising both antibodies and helper T cells. Moreover, recent studies have shown some conventional pro-inflammatory adjuvants can also promote expansion of immunosuppressive regulatory T cells (Treg) and myeloid derived suppressor cells (MDSC), both of which could negatively impact malaria disease progression. Herein, we explore the ability of a model nanoparticle delivery system (polystyrene nanoparticles; PSNPs), previously proven to not induce conventional inflammation, Treg or MDSC, to induce immunity to MSP4/5 from *Plasmodium yoelii*, a member of the MSP4 and MSP5 family of proteins which are highly conserved across diverse malaria species including *P. falciparum*. The results show PSNPs-MSP4/5 conjugates are highly immunogenic, inducing immune responses comprising both T helper 1 (Th1) and Th2 cellular immunity, and a spectrum of antibody subclasses including IgG1, IgG2a, and IgG2b. Benchmarked against Alum and Complete Freund's Adjuvant (CFA), the immune responses that were induced were of comparable or higher magnitude, for both T cell frequencies by ELISpot and antibody responses in terms of ELISA end titer. Importantly, immunization with PSNPs-MSP4/5 induced partial protection against malaria blood-stage infection (50–80%) shown to be mechanistically dependent on interferon gamma (IFN-γ) production. These results expand the scope of adjuvants considered for malaria blood-stage vaccine development to those that do not use conventional adjuvant pathways and emphasizes the critical role of cellular immunity and specifically IFN-γ producing cells in providing moderate protection against blood-stage malaria comparable to Freunds adjuvant.

Keywords: blood-stage, malaria, nanoparticles, adjuvant, immunogenicity, protection

INTRODUCTION

Malaria remains a global health problem, affecting over 200 million people annually with ∼40,000 deaths (1). It is caused by the plasmodium parasite, of which *Plasmodium falciparum* (*P. falciparum*) and *Plasmodium vivax (P. vivax)* are the most widespread. There is an urgent need for the development of an effective and long-lasting malaria vaccine. The most advanced pre-erythrocytic stage vaccine RTS, S provides <40% protection against disease in African populations (1, 2). Since any break-through parasites progressing to the blood-stage sustain transmission, pre-erythrocytic vaccines alone may be insufficient to support complete protection or eradication. Additionally, the blood-stage of malaria is symptomatic of disease, highlighting the need to eradicate parasites at or before this stage.

One of the major challenges to designing an effective vaccine for malaria is the identification of appropriate antigen targets. Many candidates are polymorphic or poorly immunogenic, thus making them inadequate vaccine antigens. One group of potential blood-stage vaccine antigens are the merozoite surface proteins (MSP) (3). MSP1, MSP2, and MSP3 have been used in human vaccine trials alone or in combination vaccines (4–6) but demonstrate little protective efficacy (6). Similar results have been seen with other leading blood-stage proteins from the merozoite, such as AMA1 which has shown promising humoral immunogenicity (7, 8) but only modest protective efficacy (9). However, there have been very few trials assessing efficacy in humans, particularly in children for blood-stage vaccines and many more are needed. Vaccine development at this stage mainly focuses on generating a neutralizing antibody response to prevent erythrocyte invasion (similar to that seen with naturally acquired immunity) though inducing strong cellular responses may be just as important to promote protection. Therefore, it is important to identify the antigenic epitopes that are not only highly immunogenic but also protective.

MSP4 and MSP5 are candidates for blood-stage vaccines as they show limited antigenic diversity in both *P. falciparum* (10–12) and *P. vivax* (13, 14). MSP5 is largely conserved across both these strains, and also geographic locations of Plasmodium (15), with polymorphisms detected only in specific gene regions (16). Naturally acquired antibodies have been detected to MSP4 (17, 18) and high antibody levels have been associated with a protective role in subsequent malaria seasons (18), though the precise cause of protection in these cohorts is yet to be fully defined. Similarly, naturally acquired antibodies against MSP4 are associated with reduced clinical malaria cases (19) and low level frequency of cross reactive antibodies have been detected to both *P. falciparum* and *P. vivax* (20). Though possibly attributed to anti-MSP antibody production, the exact correlates of protection in these studies are yet to be identified, although it does indicate these antigens are good candidates for inclusion in vaccines. Importantly, functional MSP5 specific T cell responses have also been observed to *P. falciparum* and *P. vivax* (21). The low antigen diversity of MSP4 and MSP5 and correlation with protection makes them attractive vaccine candidates compared to the large number of other highly polymorphic antigens. In animal studies, the murine equivalent homolog of MSP4 and MSP5, MSP4/5, has shown protection using different vaccine formulations (22–25), and protection is enhanced when administered in combination with MSP1 (26). Most of these studies examine antibodies in the protective response, with less known about the protective role of the cellular immune response. One study showed that the selected adjuvant AFCo1 (synthetic cochleate structures) enhanced both antibodies and T cell responses against MSP4/5, attributable to the induced Th1-like immune responses (27). Thus, it is important for vaccine design to not only aim to induce antibody responses, but also cell mediated immunity.

One possible reason for the low protective responses seen in recent malaria vaccine trials may be due in part to adjuvant or vaccine delivery platform selection. Optimal antigen and adjuvant combinations are imperative for vaccine efficacy. Alum is currently one of the only licensed adjuvants for widespread human use (28) and the RTS, S vaccine in humans contains the proprietary adjuvant AS01, consisting of liposomes, monophosphoryl lipid A (MPLA, the non toxic derivative of LPS) and the saponin QS-21 (29). Both these adjuvants, as well as most adjuvants in development for human use, are pro-inflammatory (inducing cytokines such as interleukin [IL-6] and/or tumor necrosis factor [TNF]) (30). Recent literature suggests that such pro-inflammatory adjuvants could also promote the subsequent expansion of regulatory T cells (Treg) and myeloid derived suppressor cells (MDSC), which may be a natural mechanism aimed to turn off excessive inflammation (31). This property may limit the persistence of immunity, as well as potentially increase the frequency of cells such as TNFR2+ Treg associated with the development of severe malaria (32). It is therefore useful to explore whether non-inflammatory adjuvants and vaccine carriers, which do not induce Treg or MDSC, can also induce malaria specific immunity of sufficient type and magnitude to protect against a malaria challenge.

Viral sized 40–50 nm polystyrene nanoparticles (PSNPs) are non-inflammatory and when used as adjuvanting antigen carriers in experimental vaccines, induce high levels of CD4+ and CD8+ T cells, as well as antibodies (33–37). Though there are many different types of nanoparticles currently being used for malaria vaccines (38, 39) [reviewed in Powles et al. (40)], PSNPs in this size range (and negatively charged) can target antigen presenting cells (APCs) in the local lymph nodes to increase uptake and presentation of the vaccine antigen, leading to long lasting protective immune responses in murine studies (33, 34, 36). These PSNPs have been shown to induce high level CD8+ T cell responses when covalently coupled with malaria liver stage peptide epitopes, however not when simply "mixing" the PSNPs with the peptide (31, 41). Moreover, they have been confirmed to be "inert" in terms of failing to activate inflammatory cytokine or MAPK/ERK mediated inflammatory pathways (37, 42) and do not induce TNFR2+ Treg or MDSC (31). These inert and biocompatible PSNPs have repeatedly shown to be safe and well-tolerated in numerous animal models at both low and high doses (33, 36, 37, 43, 44), and can be coupled to different protein and peptide antigens with high efficiency and loading capacities (31, 37, 44). Additionally, recent pre-clinical studies progressing the translation of this approach as a cancer vaccine have seen no

toxic or inflammatory effects when injecting similar PSNP doses in mice for up to 4 times at weekly intervals (44). Whilst the exact clearance mechanism of these PSNPs is unknown, it is known that nanoparticles in this viral size range allow their excretion from the body via hepatobiliary elimination and ultimately via feces (45).

Herein we assess both the immunogenicity and protective efficacy of blood-stage malaria vaccines in an animal model of malaria, formulated using PSNP antigen carriers using MSP4/5 as the target antigen and compared to formulations with MSP4/5 adjuvanted with Alum or the experimental adjuvant Complete Freund's Adjuvant (CFA).

MATERIALS AND METHODS

Mice

Six-to-eight-week-old BALB/c and IFN-γ gene knock out (KO) mice were purchased from the Austin Research Institute or Monash Animal Services. Austin Health and the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committees approved the use of all animals and procedures.

Recombinant MSP4/5 Production

Recombinant MSP4/5 generated in *E. coli*, described in Kedzierski et al. (22), was kindly provided by Professor R. Coppel for use in these studies. The protein concentration of recombinant MSP4/5 was determined by Bicinchoninic Assay (BCA, Thermo Fisher) following manufacturer's instructions.

Conjugation of MSP4/5 to PSNPs

Conjugation of MSP4/5 to PSNPs was performed as described previously (35, 37). Briefly, 40–50 nm carboxylated polystyrene nanoparticles (PSNPs, Polysciences Inc, USA) at a final 1% solids ($\sim 1.9 \times 10^{14}$ PSNPs/ml, unless otherwise stated) were pre-activated using a 2-*N*-morpholino-ethanesulfonicacid buffered (MES; 50 mM final, pH 6.2) solution of 1-ethyl-3-(3-dimethylaminopropryl) carbodiimide hydrochloride (EDC; 4 mg/ml final) (Sigma Aldrich) (with sulfo-NHS for some conjugations) for 15 minutes on a rotating wheel at room temperature (RT). MSP4/5 was added (target final concentration of 0.4 or 1 mg/ml) and further incubated for 2–3 hours (h) at RT. The conjugation reaction was stopped by the addition of glycine (7 mg/ml final) for a further 30 minutes at RT. Conjugation mixture was dialyzed using 100–300 kDa dialysis membrane overnight against phosphate buffered saline (PBS) at 4°C. Dialysis against 100–300 kDa cutoff membrane permits retention of the PSNPs conjugated to the antigen, and any remaining activating agents and unconjugated protein [MSP4/5 is ∼36kDa (24)] are dialyzed out. This process also allows for buffer exchange into a physiological solution, such as PBS, for immunizations. Conjugated PSNPs were stored at 4°C and sonicated for 15 minutes before use to create a uniform and homogenous formulation for immunizations.

Vaccine Formulations and Immunizations

Mice were immunized intradermally (id) once or twice with the following vaccine formulations: 100 μl of either PSNPs conjugated to MSP4/5 or MSP4/5 with adjuvant, 2–3 weeks apart for immunogenicity studies. Adjuvanted vaccine formulations contained either Alum (Rehydragel HPA, General Chemical) at 0.3 g/ml with MSP4/5 in PBS, or a prime with MSP4/5 emulsified in CFA at a 1:1 v/v ratio and boost with Incomplete Freund's Adjuvant (IFA) at a 1:1 v/v ratio. Ten to fourteen days following the last immunization, mice were humanely sacrificed by CO_2 asphyxiation. Conjugated and adjuvanted MSP4/5 protein doses, immunization volume and route were matched per experiment, with specific vaccine formulations provided in each of the figure legends. For challenge experiments, mice were injected with parasitized red blood cells (pRBCs) 3 weeks following the last immunization (see detailed procedure below).

ELISpot

Splenocytes from immunized animals were isolated from 10 to 14 days following the last immunization and assessed by ELISpot for IFN-γ and IL-4 production. Ninety six well multiscreen plates (MAHA, MAIP or MSIP, Millipore) were coated overnight at 4°C with 5 μg/ml anti-mouse IFN-γ (AN18, Mabtech) or anti-mouse IL-4 (BVD4-1d11, Mabtech or BD Biosciences). All wells were washed 5 times with PBS and blocked with RPMI 1640 (Gibco, Life technologies) containing 10% FBS (further supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, 1 M Hepes, and 0.1 mM 2- mercaptoethanol, to make complete media) for 2 h at 37°C. Splenocytes were added at a final concentration of 0.5×10^6 or 1×10^6 cells per well and co-incubated with recall antigens (MSP4/5 at 15–25 μg/ml final for each recall antigen) in complete media for 12–16 h at 37°C. Media alone and positive control Concanavalin A (1 μg/ml final) wells were also added. Following incubation, plates were washed with PBS (MSIP plates) or PBS containing 0.05% Tween 20 (MAHA and MAIP plates) and 1 μg/ml anti-mouse IFN-γ-biotin (R4-6A2, Mabtech) or anti-mouse IL-4-biotin (BVD6-24G2, Mabtech or BD Biosciences) in 0.5% FBS/PBS was added for 2 h at RT. Plates were washed and 1 μg/ml Streptavidin-ALP or extravidin-ALP in 0.5% FBS/PBS was added for a further 1–2 h at RT. Plates were given a final wash with PBS followed by reverse osmosis water and spots were developed using an AP colorimetric kit (Bio-Rad). Once plates were dry, spots were counted using an AID ELISpot Reader system (AutoImmune Diagnostika, GmbH, Germany).

ELISA

Sera from immunized animals was collected at endpoint and assessed for antigen specific antibody production by ELISA. Ninety six well plates (polyvinyl chloride micro-titer plates, Costar, or Nunc Maxisorp plates) were coated with 5 μg/ml of MSP4/5 in carbonate/bicarbonate coating buffer overnight at 4°C. Plates were washed with 0.05% Tween 20/PBS and blocked with 2% bovine serum albumin (BSA)/PBS or 5% skim milk/PBS and incubated for 1 h at 37°C. Plates were washed as above and serial dilutions of sera added and incubated for 2 h at 37°C (or 4°C overnight). Plates were washed and horseradish peroxidase (HRP) conjugated anti-mouse total Ig or IgG antibodies (1/2000 dilution for total IgG/A/M, Zymed, and sheep anti-mouse IgG, Amersham Biosciences, UK) added for 1 h at 37°C. Where indicated HRP conjugated subclass antibodies were used as

the secondary antibody and incubated for 1 h at 37°C (HRP-IgG1 at final 1/1,000, HRP-IgG2a at final 1/1,000 and IgG2b-biotin at final 1/250 dilution, BD Biosciences). Biotinylated secondary antibodies were followed with a further incubation of streptavidin-HRP (1/1,000, Amersham biosciences) for 1 h at 37°C. Plates were given a final wash and antibody detection was developed using ABTS or TMB substrate solution before being stopped with 1 M HCl. Absorbance was read at optical density (OD) 405–450 nm on a plate reader (Fluostar Optima, BMG lab technologies, or Multiscan GO, Thermo Fisher). Antibody end titers were calculated closest to the serum dilution at which the OD was equal to the mean of the naïve sera (averaged across naïve mice for all dilutions tested) plus 3 standard deviations.

Malaria Infection Challenge

Plasmodium yoelii parasites were cultured *in vivo* in naïve mice and pRBCs harvested when mice reached a required level of parasitemia (∼4–5%). pRBCs were harvested from infected mice and experimental mice were infected with 5×10^4 or 5×10^5 pRBCs in PBS by intraperitoneal injection (total volume 100 μl, 50 μl either side of abdomen). Blood parasitemia levels were monitored every 1-2 days beginning from day 3 until ∼21–25 days post-infection. Blood parasitemia levels were calculated by venous blood smears that were Giemsa stained and counted using a light microscope. A representative field at 100X magnification was chosen and the number of pRBCs including all ring, merozoite, and schizont stages calculated compared to the total number of RBCs in the field. Mice were humanely euthanized when blood parasitemia levels reached >70%, according to ethical approval, and parasitemia levels and number of surviving mice (% survival) at each time point calculated.

Statistics

Statistical analysis was performed as follows; One or two-way ANOVA with *post hoc* Tukey analysis was used to assess differences between groups for immunogenicity (ELISpot and ELISA) assays. Kaplan Meyer survival curves with log-rank (Mantel-Cox) test comparisons were used for survival analysis. All statistical analyses were performed using Graphpad Prism software (v.7.02) and statistical significance was set at $p < 0.05$. Data is expressed as mean ± standard deviation (SD) for each group, with group sizes and additional information indicated in the relevant figure legends.

RESULTS

Highly Immunogenic Vaccine Formulations of PSNPs Conjugated to MSP4/5 (PSNPs-MSP4/5) Induce Broad Immune Responses Including Th1 and Th2 Cells and Multiple Antibody Subclasses

Identifying an immunogenic and highly conserved protein for blood-stage malaria remains a challenge for vaccine development. MSP4 and MSP5 are promising blood-stage vaccine candidates in humans, as they are relatively conserved in different strains of Plasmodium compared to other highly polymorphic antigens. The murine equivalent *P. yoelii* MSP4/5 has been identified as a protective blood-stage antigen in prior murine studies (23, 25, 46), therefore, we wanted to assess this protein using our nanoparticle delivery system.

To assess the immunogenicity of PSNPs-MSP4/5 we compared this vaccine to one containing MSP4/5 mixed with the most widely used adjuvant, Alum. BALB/c mice were immunized twice intradermally at the base of tail and 14 days following the last immunization T cell (from whole splenocytes) and serum antibody responses were measured by ELISpot and ELISA, respectively. PSNPs-MSP4/5 immunization elicited high levels of antigen specific IFN-γ producing T cells, significantly higher than mice immunized with Alum and naïve controls (**Figure 1A**, $p < 0.0001$). Whilst Alum did not induce significantly higher IFN-γ producing T cells above control conditions, both the PSNPs-MSP4/5 and Alum + MSP4/5 groups induced similar levels of IL-4 producing T cells compared to naïve mice (**Figure 1B**, $p < 0.001$, $p < 0.01$, respectively). Alum is known to induce Th2 biased responses which is reflected in the above results. Furthermore, when looking at total antibody levels induced by vaccination, both Alum and PSNPs induced comparable levels of total Ig (**Figure 1C**) and similar levels of IgG1 antibodies (**Figure 1D**), the latter being classically associated with a Th2 response. Mice immunized with PSNPs-MSP4/5 developed higher IgG2a and IgG2b antibodies compared to either the Alum + MSP4/5 or the naïve groups but these responses were not significant (**Figures 1E, F**). These results indicate PSNPs induce a spectrum of IgG antibody subclasses as well as both IFN-γ-producing Th1 and IL-4-producing Th2 cells.

Comparable Immunogenicity of PSNPs-MSP4/5 Nanovaccines Against "Gold Standard" Experimental Vaccine Formulations of MSP4/5 in CFA/IFA

The immune responses generated by PSNPs-MSP4/5 immunizations were comparatively similar for Th2, and better for Th1 type responses, compared to the conventional adjuvant Alum. Therefore, we further tested the robustness of these responses against the strong experimental Th1 inducing adjuvant, CFA, followed by a boost with IFA, and compared against one or two immunizations with PSNPs-MSP4/5. MSP4/5 in CFA/IFA induced functional IFN-γ and IL-4 producing T cells, as well as IgG antibodies, significantly higher than naïve mice (**Figures 2A–C**, $p < 0.0001$, $p < 0.001$, $p < 0.01$, respectively). One immunization with PSNPs-MSP4/5 was comparable to MSP4/5 in CFA/IFA for both IFN-γ and IL-4 producing T cell immune responses (**Figures 2A,B**), and significantly higher than naïve responses (**Figures 2A,B,** $p < 0.0001$). Two immunizations with PSNPs-MSP4/5 elicited the highest levels of IFN-γ and IL-4 T cell responses, significantly higher than all other groups for IFN-γ producing T cells (**Figure 2A**, $p < 0.0001$ compared to naïve, $p < 0.001$ compared to CFA/IFA and $p < 0.01$ compared to one immunization with PSNPs-MSP4/5) and significantly higher than MSP4/5 in CFA/IFA and naïve groups for IL-4 producing T cell responses (**Figure 2B**, $p < 0.0001$ compared to naïve and $p < 0.01$ compared to CFA/ IFA). Antibody titers were also different when comparing one vs. two immunizations with PSNPs-MSP4/5. Two immunizations of PSNPs-MSP4/5 elicited

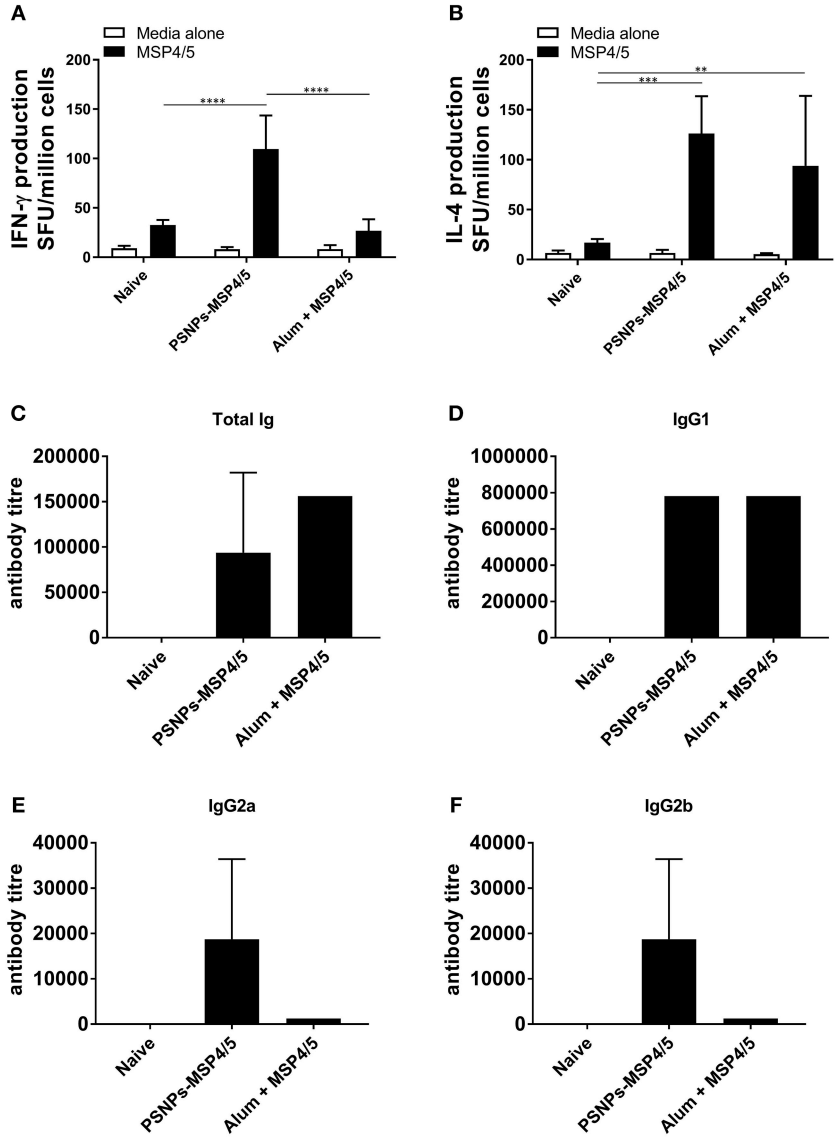

FIGURE 1 | Diverse Th1 and Th2 associated immunogenicity induced by PSNPs-MSP4/5 compared to Alum adjuvanted vaccines. BALB/c mice were immunized twice intradermally at the base of tail (100 μl/immunization), 14 days apart with PBS (Naive), PSNPs conjugated to MSP4/5 (PSNPs-MSP4/5, 40 μg/ml) or MSP4/5 in Alum (Alum + MSP4/5, 0.3 g/ml Alum and 40 μg/ml MSP4/5 in PBS). Fourteen days after the last immunization, mice were humanely sacrificed and splenocytes and serum collected for immunogenicity analysis. **(A)** IFN-γ and **(B)** IL-4 producing T cells were assessed by ELISpot and **(C)** Total IgG/A/M, **(D)** IgG1, **(E)** IgG2a, and **(F)** IgG2b anti-MSP4/5 antibody levels were measured by ELISA. Data is shown as mean spot forming units (SFU) ± SD for ELISpot and mean antibody titer ± SD of duplicate measurements of pooled sera per group for ELISA. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by two-way ANOVA with *post hoc* Tukey analysis. $n = 4$ mice per group.

higher antibody titers compared to one immunization with PSNPs-MSP4/5 (**Figure 2C**, not significant) and comparable antibody titers to MSP4/5 in CFA/IFA (**Figure 2C**). MSP4/5 in CFA/IFA induced a significantly higher antibody end titre compared to only one immunization of PSNPs-MSP4/5 (**Figure 2C**, $p < 0.05$).

Immunization with PSNPs-MSP4/5 showed strong T cell and antibody responses, especially following a two-immunization regime, therefore, we next determined the effect of PSNP dose on the resulting immune response. De-escalating doses of PSNPs (2.1×10^{14}, 3.7×10^{13}, and 1.7×10^{13} PSNP/ml) conjugated to matched levels of MSP4/5 were used to assess the potential for dose-sparing of the vaccine preparation, for both immunogenicity and protection studies. As IFN-γ has been identified as the key protective cytokine (47), the effect of different doses of PSNPs (conjugated to the same amount of MSP4/5) were assessed for their ability to affect the resulting IFN-γ T cell response. De-escalating doses of the PSNPs were compared to CFA/IFA adjuvated formulations for IFN-γ T cell responses following two immunizations. As

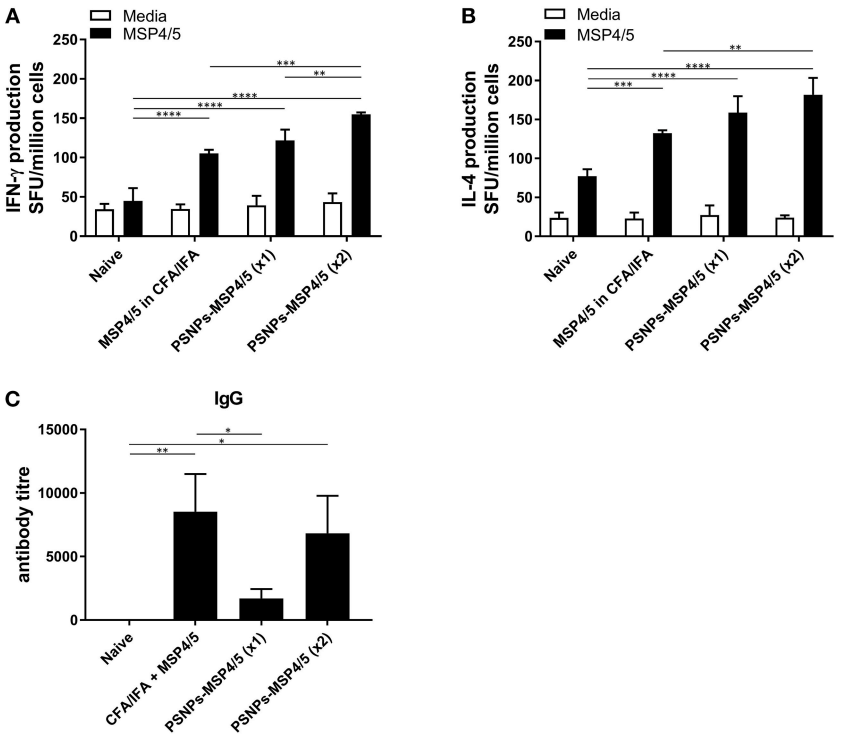

FIGURE 2 | Immunogenicity of PSNPs-MSP4/5 is comparable to CFA/IFA adjuvanted vaccines. BALB/c mice were immunized intradermally (100 μl per immunization) with one (PSNPs-MSP4/5 x1) or two (PSNPs-MSP4/5 x2) immunizations of PSNPs-MSP4/5 (100 μg/ml), or MSP4/5 in CFA (100 μg/ml MSP4/5 mixed 1:1 v/v in CFA) followed by MSP4/5 in IFA (100 μg/ml MSP4/5 mixed 1:1 v/v in IFA), or twice with PBS alone (Naïve). Ten days after the last immunization, mice were humanely sacrificed and splenocytes and serum collected for immunogenicity analysis. **(A)** IFNγ and **(B)** IL-4 producing T cells were assessed by ELISpot assay and **(C)** Total IgG antibody levels were measured by ELISA. Data is shown as mean SFU ± SD per group for ELISpot and mean antibody titer ± SD per group for ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by two-way ANOVA for ELISPOT and one-way ANOVA for ELISA analyses. $n = 6–8$ mice per group.

expected, MSP4/5 in CFA/IFA produced significantly higher T cell responses compared to controls (**Figure 3**, $p < 0.05$ compared to MSP4/5 alone and naïve). The three doses of PSNPs-MSP4/5 elicited differing IFN-γ T cell responses. Both the highest and lowest PSNP doses (2.1×10^{14} and 1.7×10^{13} PSNPs/ml-MSP4/5) induced IFN-γ producing T cell responses comparable to CFA/IFA adjuvanted vaccines and both significantly higher than control groups (**Figure 3**, $p < 0.0001$ 2.1×10^{14} PSNPs-MSP4/5 compared to MSP4/5 alone, $p < 0.001$ compared to naïve, $p < 0.001$ 1.7×10^{13} PSNPs-MSP4/5 compared to MSP4/5 alone, $p < 0.01$, compared to naïve). Unexpectedly, both the highest and lowest PSNP doses also induced significantly higher IFN-γ T cell responses compared to the 3.7×10^{13} PSNPs-MSP4/5 dose (**Figure 3**, $p < 0.01$ and $p < 0.05$, respectively), indicating no clear titratable effect of the PSNPs dose.

Immunization With PSNPs-MSP4/5 Induces Survival Against Blood-Stage Malaria Infection

Not only is it important to identify vaccine formulations that elicit strong T cell and antibody responses, they also need to translate into a clinical effect of blood-stage protection and/or enhanced survival. To investigate this, mice were immunized twice, 3 weeks apart with de-escalating doses of PSNPs-MSP4/5 and MSP4/5 in CFA/IFA. Two weeks following immunizations, mice were then challenged with *P. yoelii* parasites and monitored for parasitemia levels and overall survival. Mice reached end point when blood smears showed over 70% parasitemia levels. All groups survived significantly longer than naïve mice (**Figure 4A**, $p < 0.001$ for MSP4/5 in CFA/IFA and $p < 0.01$ for all doses PSNPs-MSP4/5, compared to naive mice) as shown on the Kaplan-Meyer survival curve. Despite observing different survival curve patterns, there were no significant differences between MSP4/5 in CFA/IFA and the three doses of PSNPs-MSP4/5 up until 21 days post-challenge, indicating these are comparably protective vaccine formulations. In terms of overall survival, the control naïve mice all reached endpoint parasitemia's (**Figure 4B**). All mice in the MSP3/5 in CFA/IFA group resolved their parasitemia levels 6/6 (**Figure 4C**), in comparison to 5/6 mice in both the 3.7×10^{13} and 1.7×10^{13} PSNPs-MSP4/5 groups (**Figures 4E,F**), and 3/6 mice in the 2.1×10^{14} PSNP-MSP4/5 group resolved their parasitemia levels (**Figure 4D**). This suggests the observed partial protection is not dependent on PSNP dose, regardless of significant differences in immune responses induced.

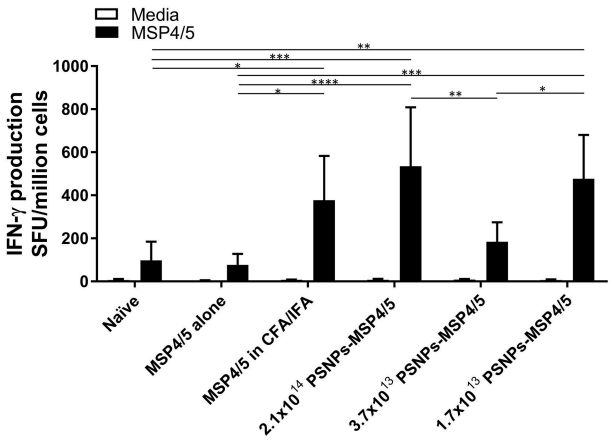

FIGURE 3 | IFN-γ producing T cell responses induced by de-escalating doses of PSNPs compared to CFA/IFA. BALB/c mice were immunized intradermally at the base of tail (100 μl/immunization) and boosted 3 weeks later with PBS alone (Naïve), MSP4/5 alone (80 μg per mouse), MSP4/5 in CFA (80 μg per mouse, 1:1 v/v with CFA) for the initial immunization and MSP4/5 in IFA (80 μg per mouse, 1:1 v/v with IFA) for the boost, or 3 different doses of PSNPs conjugated to MSP4/5 (2.1×10^{14}, 3.7×10^{13}, 1.7×10^{1} PSNPs/ml, 80 μg MSP4/5 per mouse). Fourteen days after the last immunization, mice were humanely sacrificed and splenocytes and serum collected for immunogenicity analysis. IFN-γ producing T cells were assessed by ELISpot. Data is shown as mean SFU ± SD per group for ELISpot $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by two-way ANOVA with *post hoc* Tukey analysis. $n = 4$ mice per group.

Moderate Blood-Stage Malaria Protection Induced by PSNPs-MSP4/5 Is IFN-γ dependent

In the above studies, high levels of IFN-γ and IL-4 producing T cells were induced by PSNPs-MSP4/5 vaccine formulations, as well as antibody responses including diverse IgG subclasses. Generally, functional antibodies have been associated with protective responses in humans for blood-stage malaria, with few studies characterizing the T cell response (48, 49). IFN-γ has been reported to be protective in prior *P. yoelii* liver stage murine malaria models (50), and *P. chabaudi* blood-stage infections (51), therefore, we wanted to assess mechanistically the necessity of IFN-γ production in protecting mice from blood-stage malaria immunized with PSNPs-MSP4/5. Wild type (WT) or IFN-γ KO (IFN-γ KO) BALB/c mice were immunized twice, intradermally, 2 weeks apart, with PSNPs-MSP4/5 and 2 weeks following the last immunization, mice were challenged with *P. yoelii* parasites and monitored for survival and parasitemia levels. As expected, naïve WT mice reached parasitemias over 70% (**Figures 5A,B**). PSNPs-MSP4/5 immunized WT mice were significantly protected in comparison to naive WT mice (**Figure 5A**, $p < 0.01$), with 3/6 mice protected up to 25 days post-infection and resolving their parasitemia levels (**Figures 5A,C**). Both the naïve and PSNPs-MSP4/5 experimental groups reached parasitemias above 70% in the IFN-γ KO mice (**Figures 5A,D,E**). There was a significant difference in survival between the PSNPs-MSP4/5 immunized WT mice compared to PSNPs-MSP4/5 immunized IFN-γ KO mice (**Figure 5A**, $p < 0.01$) suggesting protection may be IFN-γ dependent. There was also a significant difference between the immunized WT mice compared to naive WT mice (**Figure 5A**, $p< 0.01$), but no significant difference between the immunized and naïve IFN-γ KO mice. Some naive IFN-γ KO mice survived for marginally longer than PSNPs-MSP4/5 immunized mice, though this result was not significant.

Furthermore, antibody responses pre-challenge demonstrated high levels of IgG1 antibodies in PSNPs-MSP4/5 immunized mice in both the WT and IFN-γ KO mice, with both inducing antibody titres significantly higher than WT naïve mice and IFN-γ KO naïve mice (**Figure 5F**, $p < 0.0001$). PSNPs-MSP4/5 immunized WT mice also induced significantly higher IgG1 antibody levels compared to PSNPs-MSP4/5 immunized IFN-γ KO mice (**Figure 5F**, $p < 0.05$). IgG2a and IgG2b induction was impaired in IFN-γ KO mice but not in WT mice when immunized with PSNPs-MSP4/5. IgG2a antibody levels elicited by PSNPs-MSP4/5 in WT mice were significantly higher than WT naïve mice and both IFN-γ KO naïve and immunized mice (**Figure 5G**, $p < 0.001$). IgG2b antibodies were also significantly higher in the WT PSNPs-MSP4/5 immunized mice compared to WT naïve and IFN-γ KO naïve mice (**Figure 5H**, $p < 0.001$) and PSNPs-MSP4/5 immunized IFN-γ KO mice (**Figure 5H**, $p < 0.01$). These results may be explained when considering IgG1 antibodies are induced by IL-4 and associated with Th2 responses and IgG2 antibodies are associated with Th1 responses a and dependent on IFN-γ.

DISCUSSION

The present study shows immune responses induced by a biocompatible nanoparticle carrier vaccine approach can induce immunity and moderate protection against blood-stage malaria comparable to the "gold standard" experimental adjuvant CFA. This finding adds to the repertoire of possible adjuvants and vaccine approaches in development, by showing that delivering a classical pro-inflammatory danger signal is not necessary to induce immune responses of sufficient magnitude, or of the type, necessary to protect against blood-stage malaria infection. We note, however, that associations of immune responses with protection do not necessarily imply causality. Nonetheless, the above results support the MSP4 and MSP5 families as candidate antigens for vaccine development when used with an appropriate delivery system.

PSNPs have repeatedly shown, in a range of disease models, to be non-inflammatory and capable of inducing robust immune responses without the addition of T helper epitopes or additional stimuli (31, 37, 42, 44, 52). Side by side comparisons of immunogenicity elicited by PSNPs-MSP4/5 showed them to be capable of producing superior cellular responses to MSP4/5 in Alum, inducing balanced Th1 and Th2 responses, as well as comparable responses to MSP4/5 in CFA/IFA vaccine formulations, the latter representing a gold standard level of immune responses in experimental models. Similar results were obtained with MSP4/5 with an alternative production system in *Saccharomyces cerevisiae* (unpublished).

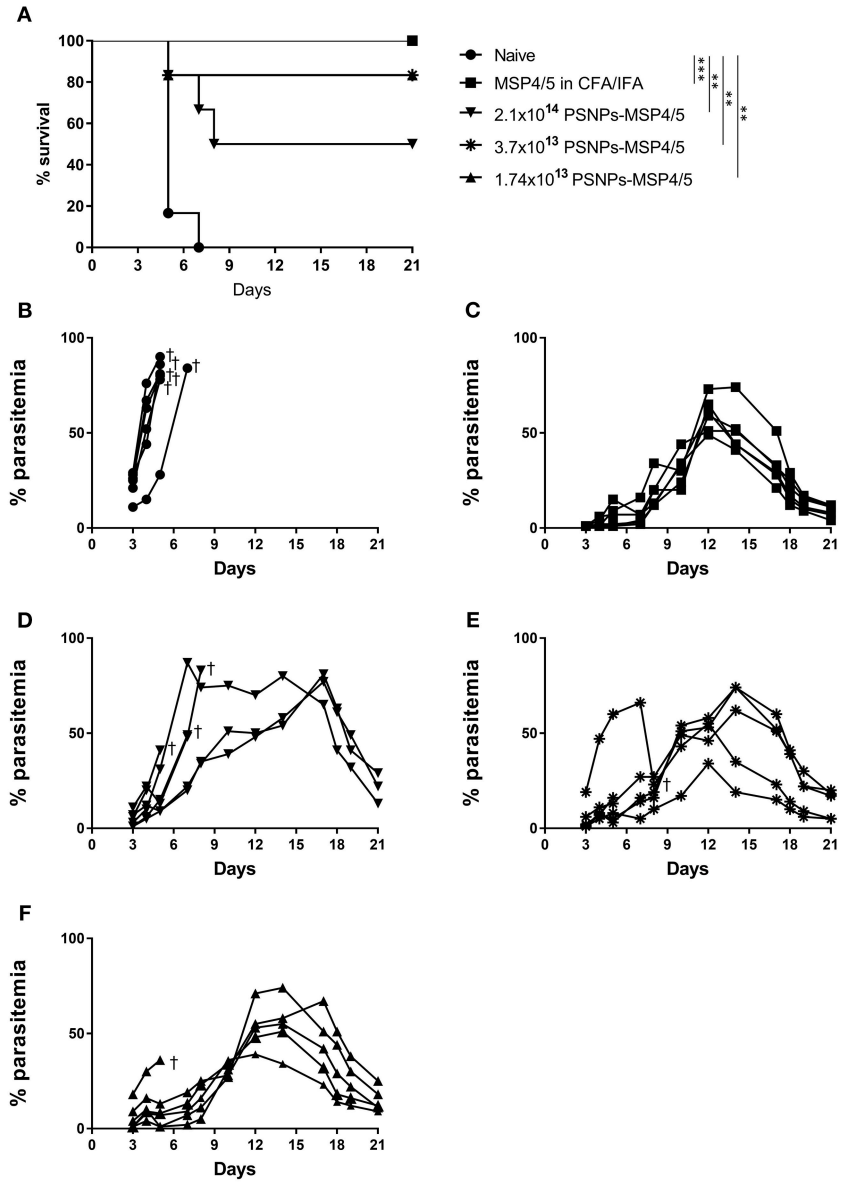

FIGURE 4 | Moderate survival against blood-stage malaria infection following immunization with de-escalating doses of PSNPs compared to CFA/IFA adjuvanted vaccines. BALB/c mice were immunized intradermally at the base of tail (100 μl/immunization) and then a boost 3 weeks later with PBS alone (Naïve), MSP4/5 in CFA (80 μg per mouse, first immunization) or MSP4/5 in IFA (80 μg per mouse, boost immunization) or 3 different doses of PSNPs-MSP4/5 (2.1×10^{14}, 3.7×10^{13}, 1.7×10^{13} PSNPs/ml). Fourteen days after the last immunization, mice were challenged intraperitoneally with 5×10^5 *P. yoelii* pRBCs and parasitemia levels were monitored starting at 3 days post-challenge. **(A)** Kaplan-Meier survival curve and percentage parasitemias of mice immunized with **(B)** Naïve -●-, **(C)** MSP4/5 in CFA/IFA -■-, **(D)** 2.1×10^{14} PSNPs-MSP4/5 -▼-, **(E)** 3.7×10^{13} PSNPs-MSP4/5 -⁎- and **(F)** 1.74×10^{13} PSNPs-MSP4/5 -▲-. $^{**}p < 0.01$, $^{***}p < 0.001$, with Mantel Cox Log Rank Test. †Indicates mouse endpoint. Data shown are from 6 mice per experimental group.

Antigen specific IFN-γ production by T cells was predominantly measured in the present study, though as whole splenocytes were used in the assays there is a possibility that IFN-γ production from other cell types (i.e. NK cells) may also be measured, but unlikely to this protein antigen. The cells that respond in ELISpot assays are predominantly T cells, as shown by depletion assays (53–55). Dose-sparing of the PSNP formulations unexpectedly showed that the middle dose induced significantly lower IFN-γ producing T cell responses, compared to the highest and lowest PSNP doses. Therefore, it will be useful in future studies to explore the dose dependency of other types of immune responses.

PSNPs-MSP4/5 formulations showed substantial improvement over MSP4/5 in Alum formulations in the elicitation of specific antibody subclasses such as IgG2a and IgG2b, but not total Ig or IgG1. Although the antibody titers induced by PSNPs-MSP4/5 vaccines were lower than the "gold standard" MSP4/5 in CFA/IFA, PSNPs-MSP4/5 vaccines

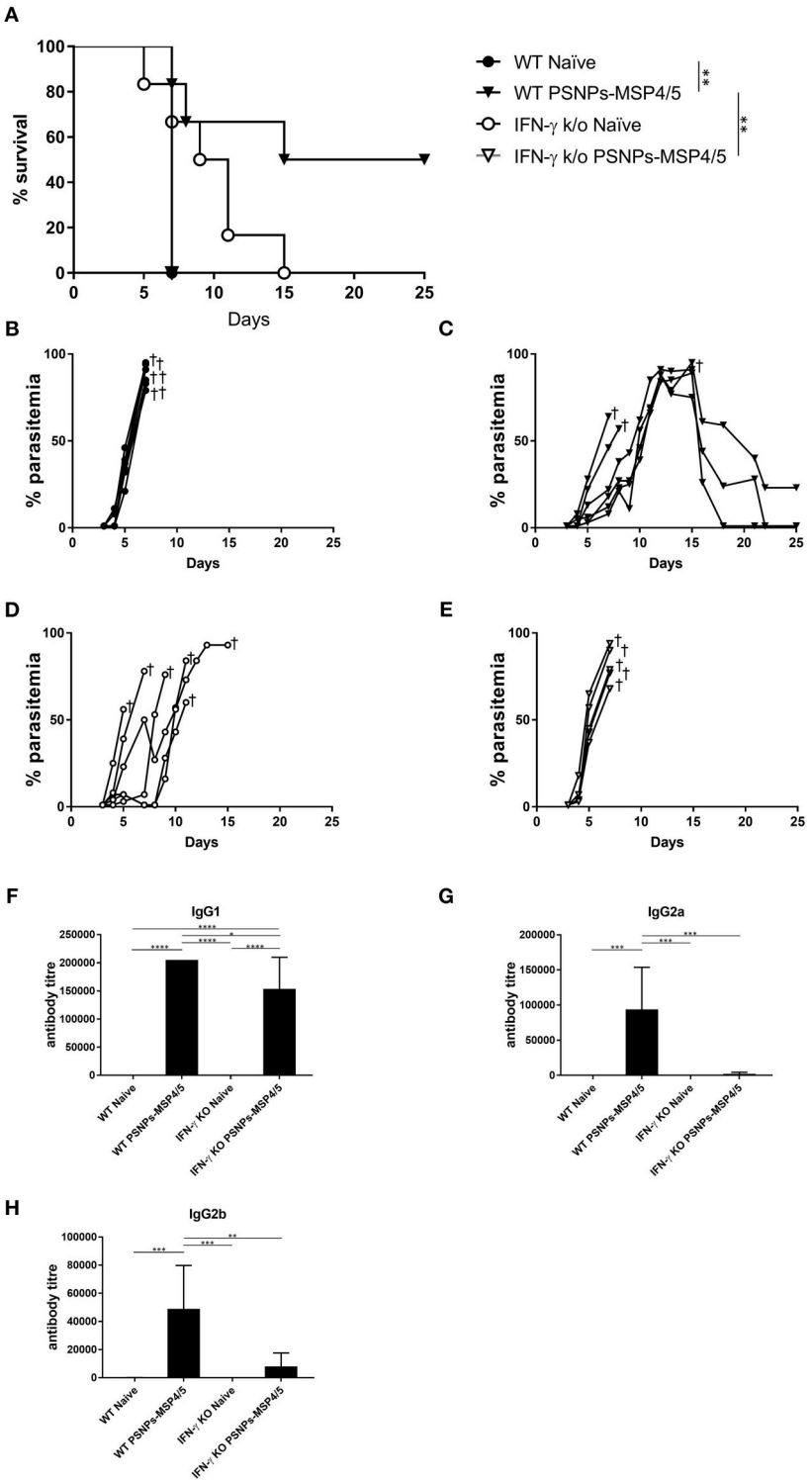

FIGURE 5 | Blood-stage malaria protection induced by immunization with PSNPs-MSP4/5 is IFN-γ dependent. BALB/c WT or IFN-γ KO BALB/c mice were immunized twice intradermally at the base of tail (100 μl/immunization), 14 days apart with PBS alone (Naïve) or PSNPs-MSP4/5 (100 μg/ml MSP4/5). Fourteen days following the boost immunization, mice were challenged intraperitoneally with 5×10^4 pRBCs from *P. yoelii* and survival parasitemia levels were monitored starting at 3 days post-challenge. **(A)** Kaplan-Meier survival curve and parasitemia levels of mice immunized with **(B)** WT Naïve -●-, **(C)** WT PSNPs-MSP4/5 -▼-, **(D)** IFNγ KO Naïve -◯- and **(E)** IFNγ KO PSNPs-MSP4/5 -▽-. Pre-challenge **(F)** IgG1, **(G)** IgG2a, and **(H)** IgG2b anti-MSP4/5 antibody levels are also shown as mean antibody titer ± SD per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ with Mantel Cox Log Rank Test for survival analysis and one-way ANOVA for antibody titer. †Indicates mouse endpoint. Data shown are from 5–6 mice per experimental group.

induced moderate protective immunity compared to CFA/IFA adjuvanted formulations. Though only CFA/IFA adjuvanted formulations achieved complete protection, it has been noted in this model that is difficult to observe complete protection (22, 24, 46). Surprisingly, there were no significant differences in the protection observed between the different PSNP doses, however there was a slightly lower survival rate in the group immunized with the highest dose of PSNPs. Overall, the partial protection observed with PSNPs in this model shows promise for current and future nanoparticle delivery platforms for blood-stage malaria.

Studying the mechanism of the protective immunity against malaria induced by PSNPs-MSP4/5, using knockout mice, demonstrated protection to be IFN-γ dependent. This is consistent with reports in the literature suggesting IFN-γ is the critical mediator of protection in erythrocytic stage infections (47), and for other strains of blood-stage Plasmodium infection (51), though the exact mechanism of protection has yet to be defined. Our results show a change in IgG subclasses elicited by the vaccine, with sustained IgG1 (Th2 associated) production but significantly decreased anti-MSP4/5 levels of IgG2a and IgG2b (Th1 associated) compared to non-immunized controls. These results indicate that the induction of IgG1 alone is not protective in this model (56), as the IFN-γ KO mice are still able to produce IgG1 but are not protected. Previous studies have shown that high levels of both IgG1 and IgG2a are required to mediate protection in the *P. yoelii* model (57, 58) however both Hirunpetcharat et al. (59) and Oakley et al. (60) showed that IgG1 alone correlates with protection against *P. yoelii* challenge (59, 60). In contrast, Adame-Gallegos et al. (61) showed that IgG1 did not mediate protection against *P. yoelii* (61).

One interesting finding in the knock out experiment was that the IFN-γ KO naïve mice had slightly better survival that the immunized KO mice (though this was not significant). Whilst it is possible that this is due to the potency of the parasites themselves, it may also be due to the differences in vaccine induced responses. As these mice do not have any vaccine induced responses, compared with the PSNPs vaccinated mice, there may be other mechanisms compensating for IFN-γ and contributing to the marginally increased survival. A potential cell type that has been identified as having a key role in clearing early parasitemia's are γδ T cells (62). It is possible that without the expansion of αβ T cells as would be expected in vaccinated animals, γδ T cells may expand instead, as well as there being a potential difference in cytokine profiles, especially from innate cells (63). Though it cannot be proven for the present study, the above hypothesis would be of interest to investigate further in this model in future studies. Indeed, in future studies it would be preferable to explore other immune responses induced by these PSNPs vaccines in the context of blood-stage malaria, including investigating other cytokines (i.e., TNF, IL-5, IL-17) in addition to the hallmark Th1 and Th2 cytokines IFN-γ and IL-4, respectively. Likewise, it would be beneficial to examine immune cell types such as B cells, γδ T cells, Th17, Tfh, Tr1, and T regulatory cells, as well as individual subsets within these populations to explore the different effector functions and cytokine profiles within cell subsets.

Plasmodium falciparum derived MSP4 and MSP5 are closely related to the MSP4/5 *P. yoelli* antigen used in this murine model of malaria. Their attractiveness as an antigen for target development came from the finding that they, and particularly MSP5, are largely conserved across malaria species, and show little polymorphism when analyzing within any single species of malaria parasites (10, 15). However, enthusiasm declined when it was shown that naturally found antibodies against MSP4 or MSP5 had little neutralizing capacity in *in vitro* red cell parasite growth inhibitory assays (GIA). Other MSP antigens were also shown to be associated with reduced incidence of malaria (64), and have been more widely studied. Although recent studies have shown association of both MSP4 and MSP5 antigens with decreased cases of malaria, specifically severe malaria (18, 19). The present study reinforces the contention that it is possible to deliver vaccine induced protection against malaria blood-stage, focusing on the induction of robust Th1 immunity, and not necessarily particular antibody functions. Of note is the fact that >60% humans naturally exposed to either acute *P. falciparum* (63%) or *P. vivax* (67%) malaria infection show IFN-γ responses to PfMSP5, suggesting it is (1) immunogenic in humans, (2) has the potential to be boosted by natural infection, and (3) is potentially a cross-reactive antigen between the two main stains of malaria found in many malaria endemic regions of the globe, including Asia (20). In agreement with previous studies, the current study suggests it is possible to design vaccines capable of inducing such potentially useful T cell responses. Moreover, we show it is possible to do so using carriers and adjuvants that do not need to deliver classical pro-inflammatory signals, a potentially important consideration when vaccinating infants in malaria endemic areas. Given our positive findings with PSNPs-MSP4/5, this supports the usefulness of further exploring other diverse nanoparticles as they are developed, as carriers for MSP antigens, beyond PSNPs. Conventional adjuvants carry the risk of damaging inflammatory responses, as well as data emerging from the literature suggesting that non-live adjuvanted vaccines may be directly associated with unhelpful non-specific effects (65). Overall, we hope the present study will spur further research into non-inflammatory vaccine carriers and adjuvants, as well as the potential for Th1 associated vaccine induced immunity in blood-stage malaria, which may help us re-examine from an additional perspective the nature of the antigens for inclusion in malaria vaccines.

AUTHOR CONTRIBUTIONS

MP designed and supervised all experiments and analyzed and interpreted all the data. KW designed and performed some of the experiments and analyzed some of the data. DP designed and performed some of the experiments and analyzed some of the data. JH and CM performed some of the experiments. SX designed and supervised some of the experiments and analyzed some of the data. RC planned some of the experiments. MP, KW, and DP wrote the manuscript and all authors contributed segments and editing to the manuscript preparation.

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Combination of RTS,S and Pfs25-IMX313 Induces a Functional Antibody Response Against Malaria Infection and Transmission in Mice

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The last two decades saw a dramatic reduction in malaria incidence rates, but this decrease has been stalling recently, indicating control measures are starting to fail. An effective vaccine, particularly one with a marked effect on disease transmission, would undoubtedly be an invaluable tool for efforts to control and eliminate malaria. RTS,S/AS01, the most advanced malaria vaccine to date, targets the parasite before it invades the liver and has the potential to prevent malaria disease as well as transmission by preventing blood stage infection and therefore gametocytogenesis. Unfortunately efficacy in a phase III clinical trial was limited and it is widely believed that a malaria vaccine needed to contain multiple antigens from different life-cycle stages to have a realistic chance of success. A recent study in mice has shown that partially efficacious interventions targeting the pre-erythrocytic and the sexual lifecycle stage synergise in eliminating malaria from a population over multiple generations. Hence, the combination of RTS,S/AS01 with a transmission blocking vaccine (TBV) is highly appealing as a pragmatic and powerful way to increase vaccine efficacy. Here we demonstrate that combining Pfs25-IMX313, one of the TBV candidates currently in clinical development, with RTS,S/AS01 readily induces a functional immune response against both antigens in outbred CD1 mice. Formulation of Pfs25-IMX313 in AS01 significantly increased antibody titres when compared to formulation in Alhydrogel, resulting in improved transmission reducing activity in standard membrane feeding assays (SMFA). Upon co-formulation of Pfs25-IMX313 with RTS,S/AS01, the immunogenicity of both vaccines was maintained, and functional assessment of the induced antibody response by SMFA and inhibition of sporozoite invasion assay (ISI) showed no reduction in biological activity against parasites of both lifecycle stages. Should this findings be translatable to human vaccination this could greatly aid efforts to eliminate and eventually eradicate malaria.

Keywords: malaria, vaccine, transmission blocking, pre-erythrocytic, synergy

INTRODUCTION

Malaria is caused by parasites of the genus *Plasmodium*, and it is one of the world's oldest and deadliest diseases. According to estimates in the World Health Organisation (WHO) world malaria report 2017 (1) the global number of malaria deaths reached 445,000 in 2016, and there were a total of 216 million malaria cases, an increase of 5 million cases over 2015. This is the first time since 2010 that case numbers increased with respect to the estimates of the previous year indicating that current control measures, which helped to drastically reduce malaria prevalence over the last decade are starting to fail and novel interventions are necessary if malaria eradication is to be achieved (2).

Vaccines are undoubtedly one of the most successful public heath interventions of all time, and played an essential role in the control or eradication of a number of diseases. An efficacious vaccine would be of immense value to all efforts to contain and eventually eradicate malaria. As outlined in the malaria vaccine technology (MVT) roadmap, as well as the malERA research agenda for malaria eradication (3, 4), an ideal malaria vaccine would have two characteristics; it would protect a vaccinated individual from illness as well as have an impact on malaria transmission (5). *Plasmodium* parasites have an exquisitely complex lifecycle with obligate developmental steps in the human host as well as in the mosquito vector. Roughly, it can be broken down in three different stages, the pre-erythrocytic stage, the erythrocytic stage or asexual blood stage and the sexual or sporogonic stage. Vaccines with an impact on malaria transmission are often referred to as VIMTs (vaccines that interrupt malaria transmission), and include classic TBVs that target the sexual life-cycle stage of the parasite in the mosquito vector as well as pre-erythrocytic vaccines (PEVs) that prevent blood stage infection and therefore gametocytogenesis. The impact of vaccines targeting the asexual blood stage, which also is the target of naturally acquired immunity, is likely to be more limited. The most advanced malaria vaccine candidate to date RTS,S/AS01 targets the pre-erythrocytic stage antigen circumsporozoite protein (CSP) of *P. falciparum*, the causative agent of the most severe form of human malaria, with the aim to interrupt the parasite's lifecycle before the establishment of liver stage infection. It is the only malaria vaccine to have completed Phase III clinical trials (6–9) and to have obtained a positive scientific opinion from the European Medicines Agency (EMA) (10). As RTS,S/AS01 targets a stage in the lifecycle before the emergence of symptomatic and transmissible stages, it has the potential to prevent disease as well as transmission. However, efficacy of RTS,S/AS01 in the recently concluded Phase III clinical trial, stayed below the targets formulated in the MVT roadmap (8, 9).

One way to build on the substantial efforts that led to development of RTS,S/AS01 is to combine it with other malaria vaccines currently in development, and indeed consensus is now that a vaccine containing multiple antigens from different life-cycle stages has the most realistic chance of success (11, 12). A recent study using two monoclonal antibodies targeting CSP and the sexual stage antigen Pfs25 at partially efficacious concentrations showed that pre-erythrocytic and sexual stage interventions synergise over multiple generations as the efficacy of PEVs is sensitively dependent on the sporozoite load of an infectious mosquito and TBVs can reduce the number of oocysts that develop in the mosquito midgut and therefore reduce the number of sporozoites that reach the mosquito's salivary gland (13). Therefore, combination of RTS,S/AS01 with a TBV, targeting the sexual stage of the parasite, is particularly attractive. Furthermore, addition of RTS,S/AS01 to a TBV could overcome one of the perceived drawbacks of TBVs, which is the absence of a direct protective effect on the vaccinee, while addition of a TBV to RTS,S/AS01 could increase the effect RTS,S has on malaria transmission, as well as reduce the emergence of escape mutants.

The most advanced candidate antigen for a transmission blocking malaria vaccine is Pfs25. It has been extensively tested in mouse models where immunization with Pfs25 containing vaccines can induce an antibody response that completely blocks parasite transmission to mosquitoes (14). Pfs25, as well as its *P. vivax* ortholog Pvs25, has been tested in clinical trials (15, 16) making it, alongside Pfs230, one of only two *P. falciparum* transmission blocking antigens to be tested in humans. This showed that antibodies against Pfs25 reduce malaria transmission in SMFA (15, 17) and that the transmission reducing activity (TRA) is correlated with the induced antibody titres. Unfortunately repeated immunizations resulted in above 50% TRA in only 9 out of 11 volunteers, and this was reduced to 2 out of 11 6 weeks later (17). Similar observations were made in early clinical trials with vaccines targeting CSP, where limited vaccine efficacy correlated with a moderate antibody response. This triggered the research and evaluation of improved vaccine candidates which eventually led to the development of RTS,S/AS01 (18–20). Consequently a number of strategies have been applied to increase the antibody response induced by vaccines targeting Pfs25 (14). These included conjugating Pfs25 to the exoprotein A of *Pseudomonas aeruginosa* (EPA), which substantially increase antibody titres against Pfs25 in mice (21), but clinical trials using Pfs25-EPA formulated in Allhydrogel found only limited and short lived serum transmission blocking activity in vacinees (16, 17). The shortcoming of Pfs25-EPA/Allhydrogel in clinical trials suggests that further antigen/adjuvant combinations need to be assessed to improve TRA of vaccines targeting Pfs25. One alternative antigen, currently under clinical development, is generated by multimerisation of Pfs25 by fusion to the nanoparticle platform IMX313 (Pfs25-IMX313). This resulted in increased antibody titres and TRA in mice after immunization, either when encoded in viral vectors or as protein in adjuvant formulations (22). Furthermore, RTS,S, the antigenic component of the RTS,S/AS01 vaccine, is delivered with a highly potent adjuvant (AS01) specifically developed to induce very high antibody responses. In this study we show that formulation of Pfs25-IMX313 with AS01 increases antibody responses against Pfs25 in mice. We also show that RTS,S and Pfs25-IMX313 can be combined without any immunological interference in mice, maintaining the level of antibody immunogenicity and functional activity induced by the individual vaccines. This is the first proof of concept of the compatibility of combining RTS,S with a TBV, which could be of great value, particularly in local malaria eradication campaigns.

RESULTS

AS01 Increases Induced Antibody Titres After Immunization With Pfs25-IMX313

Pfs25-IMX313 was purified and expressed as previously described (22), formulated in adjuvant and administered to mice, to assess whether the antibody response induced after vaccination with Pfs25-IMX313 could be improved further by using AS01 instead of Alhydrogel as the adjuvant. Three doses of Pfs25-IMX313 were tested in order to measure if there was a dose sparing effect. Ten CD-1 mice per group were immunized on day 0 and day 28 (**Figure 1A**). Blood samples were collected at day 27 and day 42 and anti-Pfs25 IgG titres assessed by ELISA (**Figures 1B,C**). On day 27 after primary immunization, only vaccine formulations containing 4 μg of Pfs25-IMX313 induced a detectable antibody response in all mice. The next lower dose, 0.4 μg of Pfs25-IMX313 in AS01 induced a detectable immune response in 9 out of 10 mice, but the induced antibody titres were significantly lower than those induced by 4 μg of Pfs25-IMX313 in AS01 ($p = 0.0118$, Dunn's multiple comparison test). The lowest dose tested, 0.04 μg of Pfs25-IMX313 in AS01, induced a detectable anti-Pfs25 IgG response only in 1 out of 10 mice, and the induced antibody titres were significantly lower than those induced by both formulations containing 4 μg of Pfs25-IMX313 (4 μg of Pfs25-IMX313 in AS01 $p < 0.0001$, 4 μg of Pfs25-IMX313 in Alhydrogel $p = 0.0006$, Dunn's multiple comparison test).

After boosting, all vaccine formulations induced a detectable immune response in all mice on day 42, while no detectable immune response was induced in the mice immunized with PBS/AS01. The two highest doses of Pfs25-IMX313 in AS01 induced the highest anti-Pfs25 IgG titres. 4 μg of Pfs25-IMX313 in Alhydrogel induced significantly lower titres than the same vaccine dose in AS01 ($p = 0.0014$). Titres induced by 0.04 μg of Pfs25-IMX313 in AS01 were significantly lower than those induced by the two higher doses in the same adjuvant (4 μg of Pfs25-IMX313 in AS01 $p < 0.0001$, 0.4 μg of Pfs25-IMX313 in AS01 $p = 0.004$, Dunn's multiple comparison test), but not than those induced by the 100-fold higher dose of 4 μg of Pfs25-IMX313 in Alhydrogel. Formulation of Pfs25-IMX313 in AS01 therefore clearly increases the vaccine's immunogenicity, but while there is a dose sparing effect when compared to formulation in Alhydrogel, the highest antibody titres are achieved by immunization with the highest tested dose.

Antibodies Induced by Pfs25-IMX313/AS01 Are Functionally Active

Functional activity of the induced antibodies after the second immunization was assessed by SMFA. Live mosquitoes were fed with a mixture of *P. falciparum* gametocyte-infected blood and purified IgG from each group, and the number of oocysts which developed in the midgut was determined after dissection (**Figure 2**). Functional efficacy was calculated as transmission reducing activity (TRA), the reduction in the number of oocysts compared to the negative control group. In a first feed, total IgG concentrations of 750, 150, and 30 μg/ml were tested. All groups apart from the one that received 0.04 μg Pfs25-IMX313/AS01 induced statistically significant TRA when compared to the negative control at the two highest IgG concentrations, but only 4 μg Pfs25-IMX313/AS01 induced statistically significant TRA at 30 μg/ml. To further dissect differences between groups at lower IgG concentrations a second feed was performed using 250, 83.3, and 27.8 μg/ml IgG. This confirmed results from the first feed with only 4 μg Pfs25-IMX313/AS01 inducing statistically significant TRA at all three IgG concentrations, suggesting the high antibody titres observed in this group correlate with improved TRA.

Adjuvant and Vaccine Dose do Not Affect Antibody Quality

As a means to assess whether differences in TRA between groups were caused by a qualitative or a quantitative difference in the antibody response, the SMFA results were correlated with the amount of Pfs25 specific IgG in the feeder (**Figure 2C**). Datasets for 4 μg Pfs25-IMX313/Alhydrogel, 4 μg Pfs25-IMX313/AS01 and 0.4 μg Pfs25-IMX313/AS01 were analyzed by linear regression. The 0.04 μg Pfs25-IMX313/AS01 group was excluded from the analyses due to the absence of any statistically significant TRA in this group. To determine the transmission reducing efficacy per anti-Pfs25 AU, anti-Pfs25 AU and vaccine groups were used as explanatory variables in a multiple linear regression analysis. The overall fit to the linear regression model was $R^2 = 0.78$, and anti-Pfs25 AU ($p < 0.001$) but not vaccine groups ($p = 0.1625$) showed a significant effect. TRA in all three groups is therefore dependent on the amount of IgG used in SMFA but not on the dose or adjuvant the vaccine was administered in, suggesting that there is no qualitative difference between the antibody responses induced by the different vaccine regimens. This, as well as the differences in the induced antibody titres measured by ELISA (**Figure 1C**), suggests that the difference in induced TRA is due to an quantitative, not a qualitative difference in the antibody response. To confirm this, a similar linear regression analysis as before was performed using the reciprocal dilution factor of the serum pool as one of the explanatory variables, instead of anti-Pfs25 AU (**Figure 2D**). This allows an indirect analysis of the antibody response's efficacy in the undiluted mouse serum from each group, therefore providing a combined assessment of antibody quantity and quality. The overall fit to the linear regression model was $R^2 = 0.68$ and the serum dilution factor ($p < 0.001$) as well as the vaccine group ($p = 0.0001$) showed significant effects. When three groups were compared, 4 μg Pfs25-IMX313/Alhydrogel and 0.4 μg Pfs25-IMX313/AS01, which had both induced a similar antibody titres also appeared to have induced a similarly efficacious response ($p = 0.0924$), while both of these vaccine formulations induced a significantly less efficacious antibody response than 4 μg Pfs25-IMX313/AS01 ($p < 0.001$ and $p = 0.0026$, respectively), which had induced a significantly higher antibody titer. This analysis therefore confirms the observation from the antibody quality analysis, that the observed difference in SMFA and the superior performance of the 4 μg Pfs25-IMX313/AS01 vaccine formulation is due to a quantitative but not a qualitative difference in the induced antibody response.

A

Group	Day 0 (Prime)	Day 27	Day 28 (Boost)	Day 42
1	4µg Pfs25-IMX313/Alhydrogel	Blood sampling for Post Prime ELISA	4µg Pfs25-IMX313/Alhydrogel	Blood sampling for Post Boost ELISA and SMFA
2	4µg Pfs25-IMX313/AS01		4µg Pfs25-IMX313/AS01	
3	0.4µg Pfs25-IMX313/AS01		0.4µg Pfs25-IMX313/AS01	
4	0.04µg Pfs25-IMX313/AS01		0.04µg Pfs25-IMX313/AS01	
5	PBS/AS01		PBS/AS01	

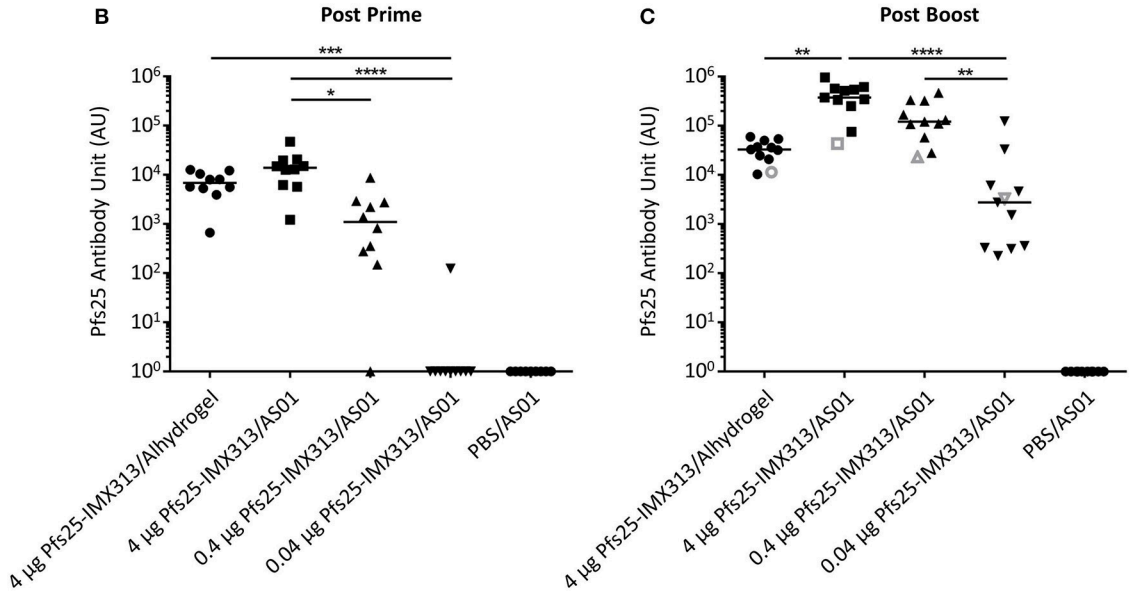


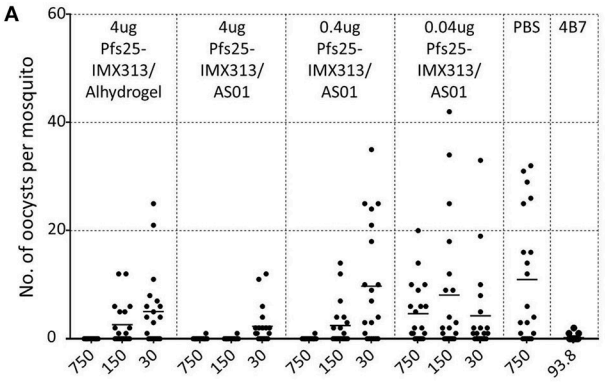
FIGURE 1 | Immunogenicity of Pfs25-IMX313 in AlhydrogelFigure or AS01. **(A)** CD1 mice ($n = 10$) were vaccinated twice with 4 µg Pfs25-IMX313 in Alhydrogel or with decreasing doses (4 µg, 0.4 µg and 0.04 µg) of Pfs25-IMX313 in AS01. BC Anti-Pfs25 IgG titres were measured by ELISA at 4 weeks post prime **(B)** and 2 weeks post boost **(C)**. Empty gray shapes show the antibody units of a 1 mg/ml dilution of purified IgG. Data points show antibody titres in individual mice, lines show the median. $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$, Dunn's multiple comparison test.

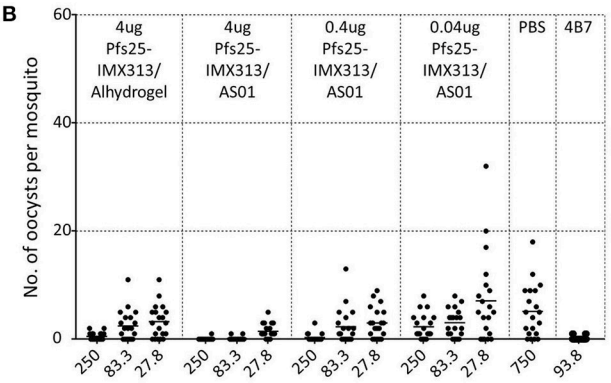
Pfs25-IMX313 can be Combined With RTS,S Without Immunological Interference

Having established that AS01 increases the immunogenicity and efficacy of Pfs25-IMX313 as well as determined an optimal dose, we assessed whether Pfs25-IMX313 could be combined with RTS,S/AS01 as a multi-antigen, multi-stage malaria vaccine. Ten CD1 mice per group were immunized on day 0 and day 28 (**Figure 3A**). The mixed vaccine group was immunized with RTS,S/AS01 and Pfs25-IMX313 combined into a single injection, while the co-administered group received both vaccines separately into the left and the right hind leg, respectively. In the latter group, each injection was formulated with only half a dose of AS01, to keep the total amount of administered adjuvant constant. Blood samples were collected at day 27 and day 42 and anti-Pfs25 and anti-CSP IgG titres were assessed by ELISA (**Figure 3**). All Pfs25-IMX313 containing vaccine formulations induced strong anti-Pfs25 antibody responses and all RTS,S containing vaccine formulations induced strong anti-CSP antibody responses. Mixing or co-administration of the two vaccines did not result in any significant reduction in antibody titres when compared to the groups that had received Pfs25-IMX313/AS01 or RTS,S/AS01 alone, suggesting that there is no immune competition between the two vaccines.

Combination of Pfs25-IMX313 With RTS,S Does not Reduce the Functional Activity of Anti-Pfs25 IgG

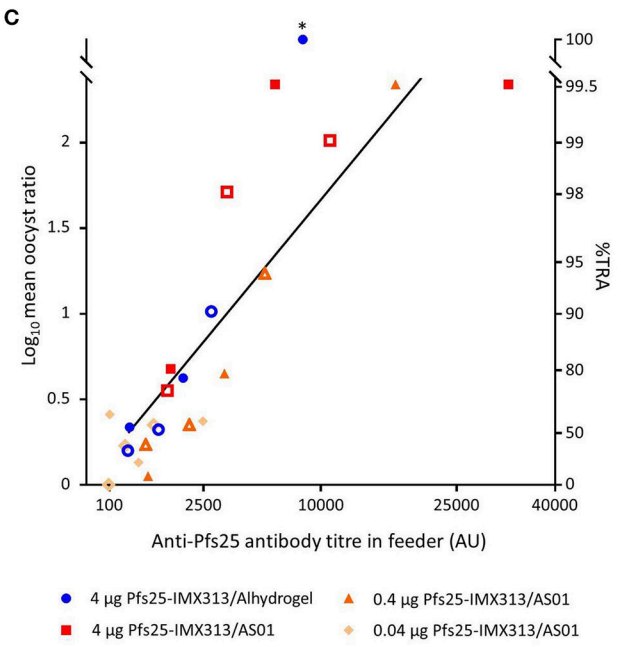
Functional activity of anti-Pfs25 IgG was assessed by SMFA (**Figure 4**). In a first feed, IgGs from all Pfs25-IMX313 containing groups were tested at 750, 250, and 83.3 µg/ml, groups that had received RTS,S/AS01 or PBS/AS01 were tested at 750 µg/ml only. Significant TRA was observed for all groups that received Pfs25-IMX313 at all the tested concentrations. Immunization with RTS,S/AS01 had no statistically significant effect on oocyst intensity. In a second feed we therefore tested lower IgG concentrations (250, 83.3, and 27.8 µg/ml) to assess for differences between groups at these lower concentrations. As





Sample	IgG conc [µg/ml][a]	Mean Oocysts[b]	% inhibition[c]	p-value[d]
4ug Pfs25-IMX313/ Alhydrogel	750	0	100 (98.8, 100)	<0.001
	150	2.6	76.3 (30.7, 91.9)	0.007
	30	5.05	53.9 (-31.6, 83.8)	0.165
4ug Pfs25-IMX313/AS01	750	0.05	99.5 (97, 100)	<0.001
	150	0.05	99.5 (98.2, 100)	<0.001
	30	2.3	79 (35.8, 93)	0.009
0.4ug Pfs25-IMX313/AS01	750	0.05	99.5 (98.4, 100)	<0.001
	150	2.45	77.6 (35.7, 92.9)	0.006
	30	9.75	11 (-165.5, 69)	0.850
0.04ug Pfs25-IMX313/AS01	750	4.65	57.5 (-30, 85.9)	0.144
	150	8.1	26 (-112.7, 74.2)	0.569
	30	4.25	61.2 (-11.7, 87.3)	0.072
PBS /AS01	750	10.95	N/A	N/A
4B7	93.8	0.2	99.1 (97.1, 99.8)	<0.001

Sample	IgG conc [µg/ml][a]	Mean Oocysts[b]	% inhibition[c]	p-value[d]
4ug Pfs25-IMX313/ Alhydrogel	250	0.5	90.3 (77.5, 96.2)	<0.001
	83.3	2.45	52.4 (-28.6, 84.4)	0.125
	27.8	3.25	36.9 (-47.7, 72.1)	0.302
4ug Pfs25-IMX313/AS01	250	0.05	99 (92.3, 100)	<0.001
	83.3	0.1	98.1 (90, 100)	<0.001
	27.8	1.45	71.8 (33.7, 88.1)	0.004
0.4ug Pfs25-IMX313/AS01	250	0.3	94.2 (85.3, 98)	<0.001
	83.3	2.3	55.3 (-16.2, 84.1)	0.083
	27.8	3	41.7 (-39.5, 76.5)	0.195
0.04ug Pfs25-IMX313/AS01	250	2.3	55.3 (-6.5, 82.2)	0.072
	83.3	3.05	40.8 (-63.2, 80)	0.297
	27.8	7.1	-37.9 (-222.4, 39.1)	0.429
PBS/AS01	750	5.15	N/A	N/A
4B7	93.8	0.2	95.2 (87.4, 98.5)	<0.001



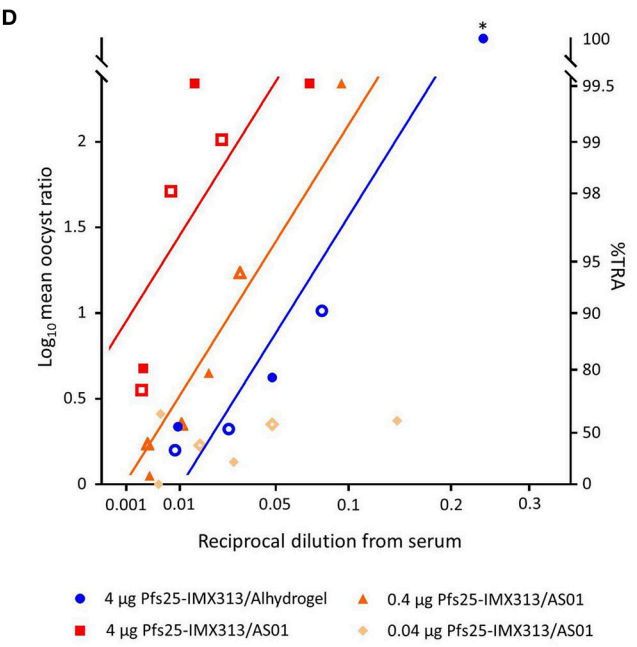


FIGURE 2 | Transmission blocking activity of anti Pfs25-IgG induced by immunization with Pfs25-IMX313 in Alhydrogel or AS01. **(A,B)** Total IgG, purified from pooled serum of each group (2 weeks post boost) was tested in SMFA in two independent experiments, one using total IgG concentrations of 750, 150 and 30 µg/ml **(A)** and one using total IgG concentrations of 250, 83.3, and 27.7 µg/ml **(B)**. The transmission blocking monoclonal anti-Pfs25 antibody 4B7 was used as a positive control. Data

(Continued)

FIGURE 2 | points represent the number of oocysts in individual mosquitoes and the lines show the arithmetic mean. X-axis values are μg/ml total IgG in the assay. The results of the two feeds are summarized in the tables. [a]IgG concentration (μg/ml) in feeder. [b]Arithmetic mean of oocyst intensity from 20 mosquitoes. [c]Percent inhibition of mean oocyst intensity and the 95% confidence interval (95% CI). [d]Two-sided *p*-values testing whether % inhibition is significantly different from zero. **(C,D)** The quality of the anti-Pfs25-IgG used in the SMFA was assessed by linear two regression analyses, correlating the Log10-transformed ratios of mean oocyst counts in control and test samples (log-mean ratio, LMR) with the anti-Pfs25 specific IgG level in the feeder **(C)** or the reciprocal dilution factor from the original serum pool **(D)**. LMR is plotted on the left y-axis. The right y-axis shows the correspondent %TRA. Values on the x-axis are plotted on a square root scale. The black line shows the shared linear fit for IgG from groups of mice immunized with 4 μg Pfs25-IMX313 in Alhydrogel or with 4 μg and 0.4 μg of Pfs25-IMX313 in AS01. Colored lines show linear fits for individual groups. Filled and unfilled shapes show data points from two independent SMFA experiments. Data points marked with an asterisk were excluded from the analysis as they showed 100% TRA (upper plateau level of dose response).

before, at IgG concentrations of 250 and 83.3 μg/ml all groups that had received Pfs25-IMX313 showed significant TRA, while immunization with RTS,S/AS01 had no statistically significant effect when compared to immunization with PBS/AS01. At the lowest tested concentration of 27.8 μg/ml, only the Pfs25-IMX313 alone group showed significant TRA. Correlation of antibody titres in the feeder with the SMFA results, however, did not indicate a difference in antibody quality between combination and individually administered vaccine (**Figure 4C**). The overall fit to the linear regression model was $R^2 = 0.68$, and again anti-Pfs25 AU ($p = 0.0012$) but not vaccine groups ($p = 0.7322$) showed a significant effect. Correlation of SMFA results with reciprocal serum dilutions furthermore confirmed the quantification of the antibody response by ELISA (**Figure 3C**) in indicating there was no quantitative difference in the IgG response between the groups ($R^2 = 0.69$, serum dilution factor: $p = 0.001$, group: $p = 0.1275$). The observed difference in SMFA is therefore most likely due to a higher concentration of Pfs25-specific IgG in the purified total IgG from the Pfs25-IMX313 alone group, than in the total IgG purified from the groups that received both vaccines ($\log_{10}$ antibody titer of 1 mg/ml purified IgG: mixed = 3.76, co-administered: 3.75, Pfs25-IMX313 alone: 4.06). This is however not reflective of the amount of Pfs25-specific IgG in serum of immunized mice, which was similar in all three groups (arithmetic mean $\log_{10}$ antibody titer: mixed = 4.83 AU, co-administered =4.81 AU, Pfs25-IMX313 alone = 4.86 AU).

Combination of RTS,S With Pfs25-IMX313 Does not Reduce the Functional Activity of Induced Anti-CSP Antibodies

Functional activity of anti-CSP antibodies was assessed by ISI assay using *P.berghei* sporozoites expressing PfCSP under the PbCSP promoter (**Figure 5**). Sporozoites were obtained from the salivary glands of infected *A. stephensi* mosquitoes and added to Huh7 hepatoma cells in the presence of test and control serum. At 1% serum concentration inhibition of sporozoite invasion is detectable in all groups that received RTS,S. There is no statistically significant difference between groups immunized with RTS,S/AS01 alone or mixed or co-administered with Pfs25-IMX313, but all groups that received RTS,S inhibit sporozoite invasion above the assay's limit of sensitivity and inhibition was significantly higher than in the group that had been immunized with Pfs25-IMX313 (mixed $p = 0.0011$, co-administered $p = 0.0154$, RTS,S alone $p = 0.0015$, Dunn's multiple comparison test). To further characterize the anti-CSP antibody response induced by the different vaccine formulations containing RTS,S the log transformed mean ratio of infected hepatocytes in the vaccinated and PBS control groups was plotted against the square root of the anti-CSP endpoint titer in the hepatocyte growth medium, as had been done to analyse SMFA results. For all vaccine groups there was a significant correlation between increased LMR and higher anti-CSP AU (one-tailed Spearman correlation, mixed $r = 0.9273$, $p = 0.0002$, co-administered $r = 0.612$, $p = 0.0334$, RTS,S alone $r = 0.8601$, $p = 0.0036$. In a multiple linear regression analysis anti-CSP AU and vaccine groups were used as explanatory variables. The overall fit to the linear regression model was $R^2 = 0.55$, and anti-CSP AU ($p < 0.001$) but not vaccine groups ($p = 0.7913$) showed a significant effect on the inhibition of sporozoite invasion, indicating that there is no loss of quality in the antibody response when RTS,S is combined with Pfs25-IMX313 rather than administered alone.

DISCUSSION

The modest efficacy of recent clinical trials using single antigen subunit vaccines support the development of multi-antigen vaccines targeting multiple stages of malaria. Mixing vaccines in a multi-component formulation is an easy process that does not require developing a new product. This means that it is possible to draw from previous experience in product development and clinical trials. This experience is particularly rich in the case of RTS,S the most advanced malaria vaccine candidate to date. Pfs25-IMX313 is a TBV candidate that we developed specifically to overcome the low antibody titres induced by vaccines targeting Pfs25 in past clinical trials (15, 17). Encoded in viral vectors it has now been tested in a clinical trial (ClinicalTrials.gov Identifier: NCT02532049), and it is currently being produced as a nanoparticle to GMP standards to be tested in clinical trials as a protein in adjuvant formulation, which in mice induced higher antibody titres than viral vectors (22). Here we showed that Pfs25-IMX313 can be formulated in AS01, which results in increased antibody titres when compared to formulation in Alhydrogel as well as increased TRA in SMFA. The functional activity of the induced antibodies per AU is similar for both adjuvants, but the overall stronger antibody response induced by Pfs25-IMX313/AS01 results in a substantially improved efficacy over Pfs25-IMX313/Alhydrogel.

This study also shows that RTS,S and Pfs25-IMX313 can be combined successfully maintaining both the antibody titres

A

Group	Day 0 (Prime)	Day 27	Day 28 (Boost)	Day 42
1	4μg Pfs25-IMX313 + 5μg RTS,S/AS01 (**mixed**)	Blood sampling for Post Prime ELISA	4μg Pfs25-IMX313 + 5μg RTS,S/AS01 (**mixed**)	Blood sampling for Post Boost ELISA and SMFA
2	4μg Pfs25-IMX313 + 5μg RTS,S/AS01 (**co-administered**)		4μg Pfs25-IMX313 + 5μg RTS,S/AS01 (**co-administered**)	
3	5μg RTS,S/AS01		5μg RTS,S/AS01	
4	4μg Pfs25-IMX313/AS01		4μg Pfs25-IMX313/AS01	
5	PBS/AS01		PBS/AS01	

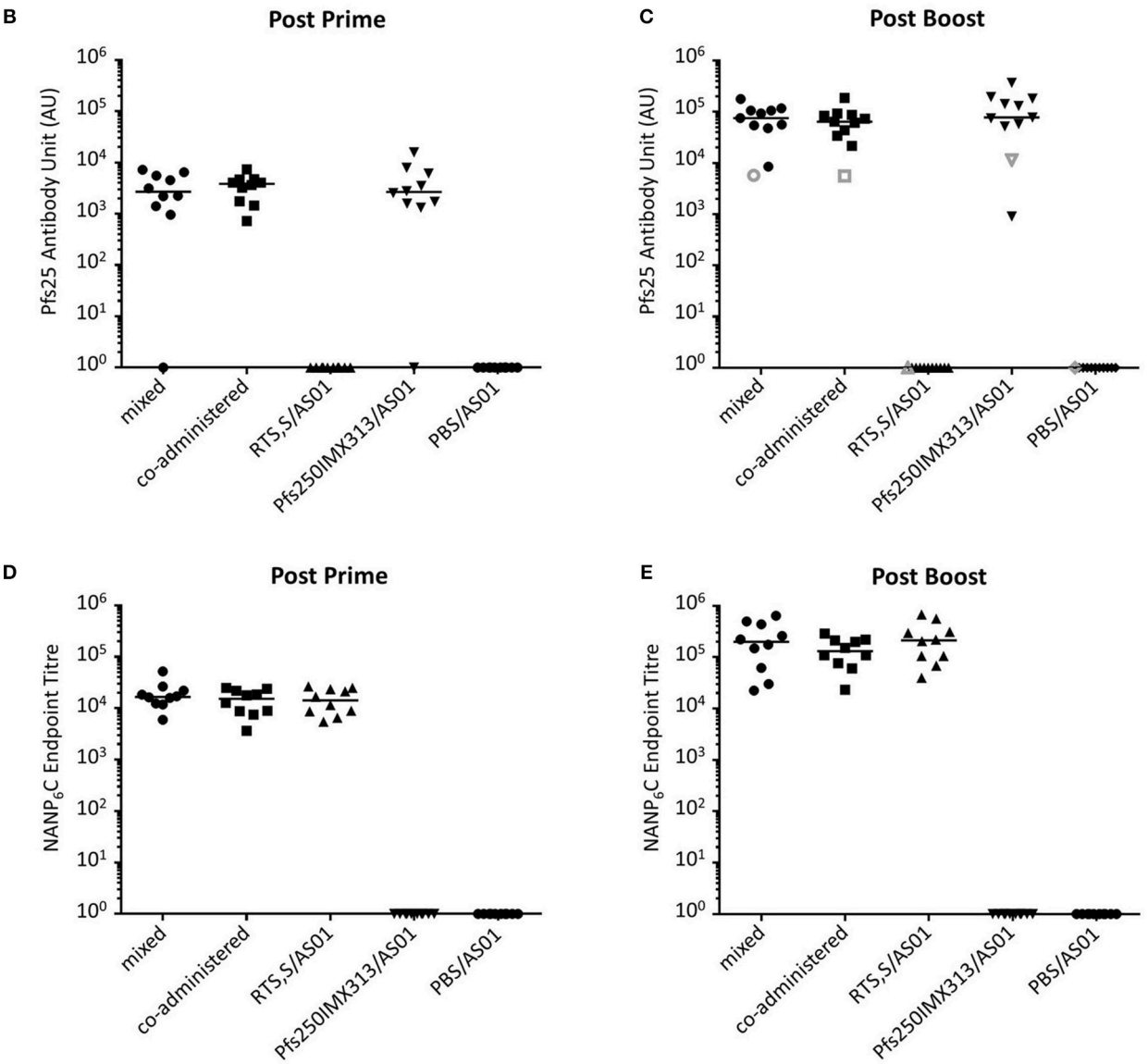


FIGURE 3 | Immunogenicity of Pfs25-IMX313 and RTS,S alone or when combined. **(A)** CD1 mice ($n = 10$) were vaccinated twice with (1) a mixture of 4 μg Pfs25-IMX313 and 5 μg RTS,S/AS01, (2) 4 μg Pfs25-IMX313/AS01 and 5 μg RTS,S/AS01, administered separately in individual hind legs, (3) 5 μg RTS,S/AS01 and (4) 4 μg Pfs25-IMX313/AS01. A control group (5) was immunized with PBS + AS01. **(B,C)** Anti-Pfs25 IgG titres were measured by ELISA at 4 weeks post prime

(Continued)

FIGURE 3 | (B) and 2 weeks post boost **(C)**. Empty shapes show the antibody units of a 1 mg/ml dilution of purified IgG. **(D,E)** Anti-NANP6C IgG endpoint titres were measured by ELISA at 4 weeks post prime **(D)** and 2 weeks post boost **(E)**. Data points show antibody titres in individual mice, lines show the median.

and functional activity of antibodies against CSP and Pfs25 compared to the individual vaccines. The perceived lower activity in SMFA of the combined vaccines is explained by the higher amount of antigen specific IgG in the total IgG preparation of the Pfs25-IMX313 alone group (**Figure 3C**), and not an inferior antibody quality in the groups that received both vaccines (**Figure 4C**). Quantification of anti-Pfs25 serum antibody titres by ELISA (**Figure 3C**) and correlation of TRA with reciprocal serum dilutions (**Figure 4D**) showed that a similar amount of anti-Pfs25 specific IgG had been generated in all three groups. However, in the groups that received RTS,S as well as Pfs25-IMX313 there is an additional strong antibody response against the CSP component in RTS,S. A fixed amount of total IgG from these groups will therefore contain a significant amount of anti-CSP antibodies not present in a total IgG preparation from mice that had been immunized with Pfs25-IMX313 alone, and hence a smaller amount of anti-Pfs25 specific IgG. A trend for increased oocyst intensity after immunization with RTS,S/AS01 (**Figure 4**) is not statistically significant, and most likely due to the high variability of the assay at lower inhibition range, rather than to an enhancing effect of RTS,S/AS01 on parasite transmission. This strongly suggests that there is no negative effect on the TRA induced by immunization with Pfs25-IMX313 when the vaccine is either mixed or co-administered with RTS,S/AS01.

The successful combination of the two vaccines contrasts with previous studies where the combination of different viral vectored malaria vaccines did result in strong immune interference (23, 24) and the combination of RTS,S/AS01 with a viral vector PEV (ChAd63/MVA ME-TRAP) failed to improve vaccine efficacy when compared to the two vaccines administered individually and reduced immunogenicity and efficacy against malaria (25). This success might be attributed to shared characteristics and modes of action of RTS,S/AS01 and Pfs25-IMX313. Both vaccines target antigens that are expressed with high abundance on the surface of the sporozoites and zygotes/ookinetes, respectively, both vaccines are produced in yeast and both vaccines use a particulate platform to array the antigen in order to induce a strong antibody response.

The combination of RTS,S/AS01 and Pfs25-IMX313, as we demonstrated here, is of particular interest, for combining a TBV with a vaccine that protects from clinical disease creates a vaccine which reduces disease as well as malaria transmission, as called for in the MVT roadmap and the malERA research agenda for malaria eradication (malERA 3; 5; malERA 4). Furthermore, a recent study in mice, using monoclonal antibodies against CSP and Pfs25, suggests that partially efficacious PEVs and TBVs could be synergistic within a population (13), as the protective efficacy of PEVs is negatively correlated with the parasite density within an infectious mosquito (26), which can be reduced through the presence of anti-Pfs25 antibodies in the human host (27). Finally, not being a target of naturally acquired immunity against malaria and therefore not under immune pressure, the sequence of Pfs25 is highly conserved (28). The selection of *P. falciparum* variants less affected by anti-CSP antibodies induced by RTS,S/AS01 can therefore likely be reduced through the presence of anti-Pfs25 which reduce the human-to-mosquito transmission of such escape variants. The advanced stage of RTS,S/AS01 and Pfs25-IMX313 as malaria vaccine candidates harbors the intriguing prospect of being able to test these hypothesis, with their potentially far reaching consequences for malaria control, in human clinical trials.

MATERIAL AND METHODS

Vaccines and Adjuvants

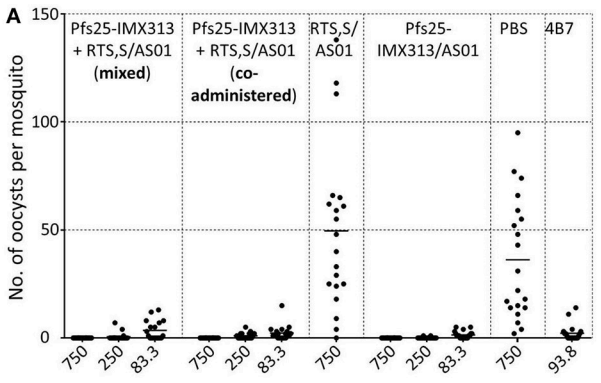
RTS,S and AS01 were provided by GSK. Pfs25-IMX313 was expressed and purified as described previously (22). In brief: Pfs25-IMX313 was expressed in *Pichia pastoris* under the control of the methanol inducible promoter AOX1. N-terminal fusion to the alpha-mating factor secretion signal sequence allowed secretion into the culture media. Following expression, nanoparticles were purified from the culture supernatant using a 5 ml CaptureSelect^{TM} C-tag affinity matrix column.

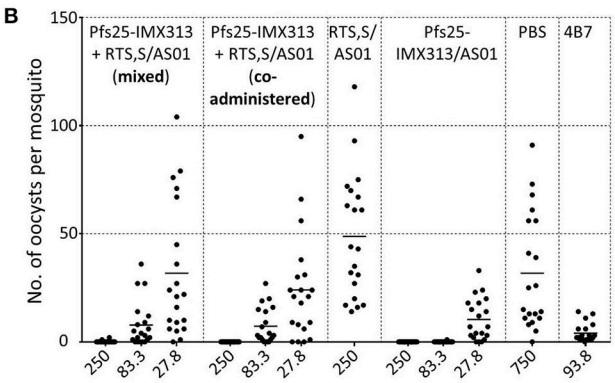
Immunizations

All animal experiments and procedures were performed according to the UK Animals (Scientific Procedures) Act Project License (PPL 30/2889) and approved by the Oxford University Local Ethical Review Committee. Age-matched female CD1 mice (Envigo, United Kingdom), housed in specific pathogen-free environments, were vaccinated with 50 μl of vaccine formulation in one leg intramuscularly (IM). Protein-in-Alhydrogel was formulated as follows: 85 μg Alhydrogel (Brenntag) per dose was mixed with TBS at room temperature for 15 min, antigen was then added and incubated for a further 60 min at room temperature. AS01E was supplied pre-mixed by GSK to be at 1-fold concentration upon mixing with an appropriate amount of antigen. Total protein per dose for each group is detailed in the tables in **Figures 1**, **3**. Animals were immunized on days 0 and 28, and blood samples were collected on days 27 (4 weeks post prime) and 42 (2 weeks post boost). The samples were allowed to clot at 4°C overnight before centrifugation at 13,000 × *g* in a benchtop centrifuge and serum was collected for testing.

ELISA

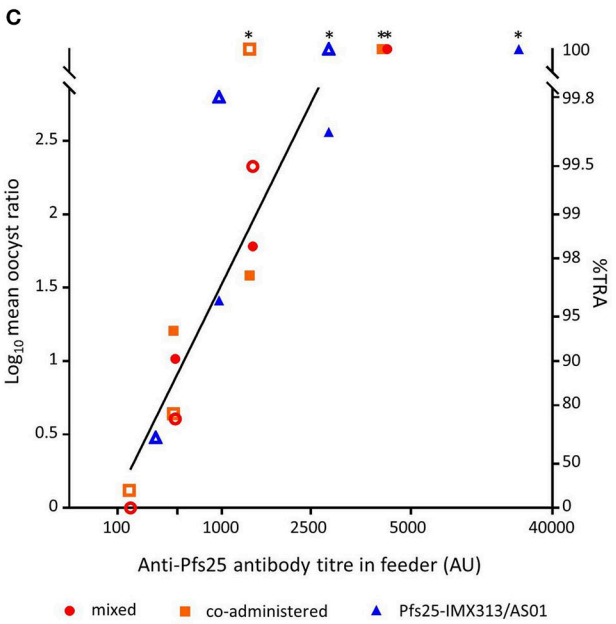
Standardized Pfs25 total IgG ELISAs were carried out as described previously (29). In brief, Nunc-Immuno Maxisorp 96 well plates (Thermo Scientific, UK) were coated with 1μg/ml Pfs25 in carbonate-bicarbonate coating buffer (Sigma Aldrich, UK) overnight at 4°C. Plates were washed with PBS-Tween and blocked with 5% milk in PBS-Tween. Sera were diluted to reach an OD405 in the linear range of the standard curve at the same time an internal control reaches an OD405 of 1. Samples were added in triplicates. An internal control was added





Sample	IgG conc [μg/ml][a]	Mean Oocysts[b]	% inhibition[c]	p-value[d]
Pfs25-IMX313 + RTS,S/AS01 (**mixed**)	750	0	100 (99.7, 100)	<0.001
	250	0.6	98.3 (96.3, 99.3)	<0.001
	83.3	3.5	90.3 (78.1, 95.8)	<0.001
Pfs25-IMX313 + RTS,S/AS01 (**co-administered**)	750	0	100 (99.8, 100)	<0.001
	250	0.95	97.4 (92.6, 99.6)	<0.001
	83.3	2.25	93.8 (85.5, 97.4)	<0.001
RTS,S/AS01	750	49.6	-37 (-223.6, 40)	0.443
Pfs25-IMX313/AS01	750	0	100 (99.6, 100)	<0.001
	250	0.1	99.7 (99.2, 100)	<0.001
	83.3	1.4	96.1 (90.9, 98.5)	<0.001
PBS/AS01	750	36.2	N/A	N/A
4B7	93.8	2.15	94.1 (83.6, 98.1)	<0.001

Sample	IgG conc [μg/ml][a]	Mean Oocysts[b]	% inhibition[c]	p-value[d]
Pfs25-IMX313 + RTS,S/AS01 (**mixed**)	250	0.15	99.5 (98.7, 99.9)	<0.001
	83.3	7.85	75.3 (41.6, 89.6)	0.003
	27.8	31.75	0 (-132.3, 56)	0.988
Pfs25-IMX313 + RTS,S/AS01 (**co-administered**)	250	0	100 (99.7, 100)	<0.001
	83.3	7.25	77.2 (49.4, 90.2)	<0.001
	27.8	24.05	24.3 (-72.3, 68.4)	0.499
RTS,S/AS01	250	48.8	-53.7 (-244.7, 30.8)	0.274
Pfs25-IMX313/AS01	250	0	100 (99.6, 100)	<0.001
	83.3	0.05	99.8 (99.6, 100)	<0.001
	27.8	10.4	67.2 (26.6, 86.5)	0.01
PBS/AS01	750	31.75	N/A	N/A
4B7	93.8	4	89.7 (76.2, 95.4)	<0.001



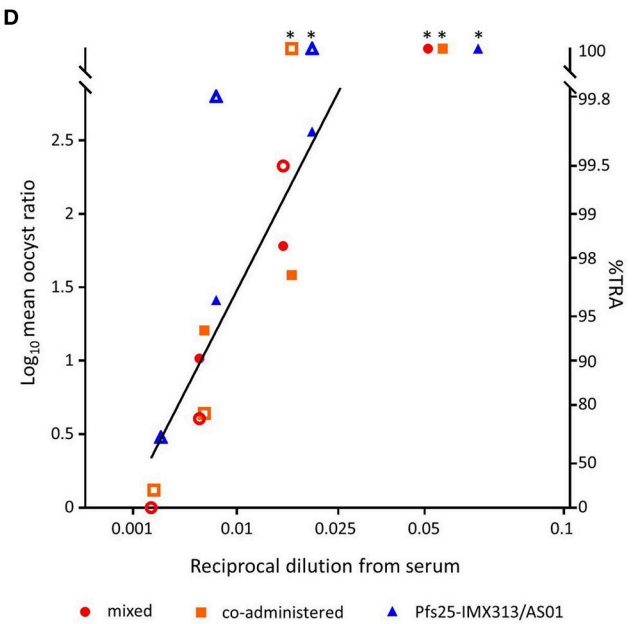


FIGURE 4 | Transmission blocking activity of anti Pfs25-IgG induced by immunization with Pfs25-IMX313 alone or in combination with RTS,S. **(A,B)** Total IgG, purified from pooled serum of each group (2 weeks post boost) was tested in SMFA in two independent experiments, one using total IgG concentrations of 750, 250 and 83.3 μg/ml **(A)** and one using total IgG concentrations of 250, 83.3, and 27.7 μg/ml. The transmission blocking monoclonal anti-Pfs25 antibody 4B7

(Continued)

FIGURE 4 | was used as a positive control. Data points represent the number of oocysts in individual mosquitoes and the lines show the arithmetic mean. X-axis values are μg/ml total IgG in the assay. The results of the two feeds are summarized in the tables. [a]IgG concentration (μg/ml) in feeder. [b]Arithmetic mean of oocyst intensity from 20 mosquitoes. [c]Percent inhibition of mean oocyst intensity and the 95% confidence interval (95% CI). [d]Two-sided *p*-values testing whether % inhibition is significantly different from zero. **(C,D)** The quality of the anti-Pfs25-IgG used in the SMFA was assessed by linear two regression analyses, correlating the Log10-transformed ratios of mean oocyst counts in control and test samples (log-mean ratio, LMR) with the anti-Pfs25 specific IgG level in the feeder **(C)** or the reciprocal dilution factor from the original serum pool **(D)**. LMR is plotted on the left y-axis. The right y-axis shows the correspondent %TRA. Values on the x-axis are plotted on a square root scale. The black line shows the shared linear fit for IgG from all groups. Filled and unfilled shapes show data points from the two independent SMFA experiments. Data points marked with an asterisk were excluded from the analysis as they showed 100% TRA (upper plateau level of dose response).

in six replicates. A standard curve was added in duplicates, starting with a 1:1,000 dilution of reference sera diluted down the plate in 1:2 steps 10 times. Plates were incubated for 2 h at room temperature and then washed as before. Goat anti-mouse whole IgG conjugated to alkaline phosphatase (Sigma Aldrich, UK) was added for 1 h at room temperature. Following a final wash, plates were developed by adding p-nitrophenylphosphate at 1 mg/mL in diethanolamine buffer (Pierce, UK) and optical density (OD) was read at 405 nm. Antibody units (AU) were defined as the dilution of a serum sample at which it had the same OD as the internal control. AUs were extrapolated from the samples OD on the linear range of the standard curve.

NANP-repeat endpoint total IgG ELISAs were carried out as described previously (30). Nunc-Immuno Maxisorp 96 well plates (Thermo Scientific, United Kingdom) were coated with 2 μg/ml NANP6C peptide in carbonate-bicarbonate coating buffer (Sigma Aldrich, United Kingdom) overnight at 4°C. Plates were washed with PBS-Tween and blocked with 10% Casein Block (Thermo Scientific, United Kingdom). Sera were diluted at a starting concentration of 1:1,000, added in duplicate, and serially diluted 3-fold. Plates were incubated for 2 h at room temperature and then washed as before. Goat anti-mouse whole IgG conjugated to alkaline phosphatase (Sigma Aldrich, United Kingdom) was added for 1 h at room temperature. Following a final wash, plates were developed by adding p-nitrophenylphosphate at 1 mg/mL in diethanolamine buffer (Pierce, UK) and optical density (OD) was read at 405 nm. Serum antibody endpoint titres were taken as the x-axis intercept of the dilution curve at an absorbance value of 0.15. A monoclonal antibody against CSP (2A10) was included in each assay as a reference control.

SMFA

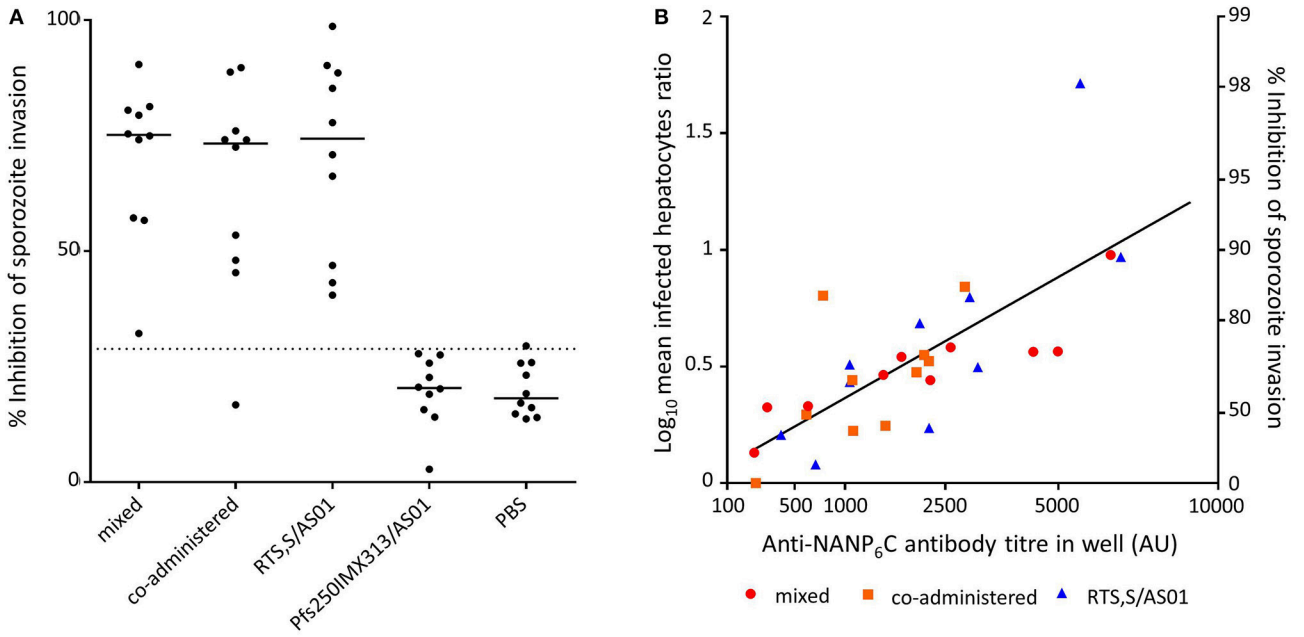
The ability of vaccine-induced antibodies to block the development of *P. falciparum* NF54 strain oocysts in the mosquito midgut was evaluated by SMFA as described previously (31). Stage V gametocytes from a mature gametocyte culture were mixed with normal human serum and normal red blood cells to make a feeding mixture with 0.15–0.2% stage V gametocytemia. Purified IgG was added to these at the concentrations shown in the figures and then fed to 3–6 day old starved female *Anopheles stephensi* (SDA 500) via a parafilm® membrane. The mosquitoes were maintained for 8 days and then dissected to count the number of oocysts per midgut in 20 mosquitos. The transmission blocking, Pfs25 specific monoclonal antibody 4B7 was used as a positive control (31). Percent reduction in infection intensity was calculated relative to the respective control IgG tested in the same assay.

Parasite Production

To produce parasites for the ISI assay, donor mice were intraperitoneally injected with a parasite bloodstock. Parasitaemia was monitored through blood sampling by tail tip amputation and thin film preparation. As soon as the parasitaemia exceeded 5%, the mouse was anesthetized by intra muscular injection of ketamine and placed on top of a pot containing approximately 50 female *A. stephensi* mosquitoes. Mosquitoes were allowed to feed through the mesh covering the pots for at least 10 min. Seven to ten days after the blood meal mosquitoes were allowed to feed on an uninfected mouse, as this had been shown to increase the number of parasites per mosquito. Twenty-one days after the initial feed salivary glands were dissected from the mosquitoes to obtain sporozoites.

ISI

Functional activity of anti-sporozoite antibodies was assessed by ISI using transgenic *Plasmodium berghei* parasites expressing PfCSP under the PbCSP promoter, at the PbCSP locus, replacing the PbCSP gene, as well as GFP to allow detection of infected cells by flow cytometry (Salman, in preparation). This strain of transgenic parasites has been validated for use in ISI (32) and in murine malaria challenge models (33). One day prior to mosquito dissection for isolation of sporozoites, HuH7 cells were seeded in a 96 well plate at a concentration of 300,000 cells/ml in a volume of 100 μl/well, resulting in a concentration of 30,000 cells per well. One day later mosquitoes were dissected and salivary glands collected in ice cold RPMI. Sporozoites were released from salivary glands by tissue disruption using a pestle and mortar, and counted in a hemocytometer. Sera were diluted to double the required concentration in 120 μl complete RPMI. The medium was removed from the HuH7 cells seeded the day before, and 50 μl of diluted serum was added to the wells in duplicates. Sporozoites were then diluted to 300,000 sporozoites per ml in complete RPMI and 50 μl were added to each well, resulting in 15,000 sporozoites per well and a ratio of cells to sporozoites of 2:1. To aid sporozoite invasion of the cells, the plate was centrifuged at 500 × g for 5 min before incubation at 37°C and 5% CO_2 overnight in a tissue culture incubator. After 24 h of incubation, cells were detached from the plate by removing medium from the plate and then adding 100 μl of TrypLE ™ Express (Gibco) to the wells and incubation for 5 min



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mouse #	Pfs25-IMX313 + RTS,S/AS01 (mixed)		Pfs25-IMX313 + RTS,S/AS01 (co-administered)		RTS,S/AS01	
	endpoint titre[a]	% inhibition[b]	endpoint titre[a]	% inhibition[b]	endpoint titre[a]	% inhibition[b]
1	5.8	90	5.0	45	5.3	47
2	4.3	32	5.3	73	5.0	66
3	4.8	57	5.3	74	5.5	71
4	4.5	57	4.8	53	4.6	43
5	5.6	75	5.2	48	5.8	90
6	5.7	81	5.0	74	5.0	78
7	5.3	74	5.3	76	5.5	89
8	5.2	75	5.5	90	4.8	40
9	5.2	79	4.4	17	5.3	85
10	5.4	81	4.9	89	5.7	99

FIGURE 5 | Inhibition of sporozoite invasion by anti CSP-serum induced by immunization with RTS,S alone or in combination with Pfs25-IMX313. **(A)** Serum from individual mice (2 weeks post boost) was mixed with *P. berghei* sporozoites from mosquito salivary gland dissections, and the inhibition of sporozoite invasion was assessed. Data points show inhibition of sporozoite invasion by serum from individual mice, lines show the median, the dotted line shows the limit of sensitivity. **(B)** The quality of anti-CSP serum induced in individual mice was assessed by linear regression analysis, correlating the anti-CSP IgG endpoint titres in the culture medium with the log-transformed ratios of infected hepatocytes in control and test samples (log-mean ratio, LMR). LMR is plotted on the left y-axis. The right y-axis shows the correspondent %ISI. Values on the x-axis are plotted on a square root scale. The black line shows the shared linear fit for IgG from all groups. **(C)** The induced anti-NANP endpoint titres and the correlating %ISI in individual mice are detailed in the table. [a]Log10-transformed NANP6C endpoint titres as determined by ELISA. [b]% Inhibition of sporozoite invasion by 1% serum in culture medium.

at 37°C and then transferred into cluster tubes. The empty wells were washed twice with 200 µl FACS buffer (PBS +0.5% BSA +0.05% Azide). Both washes were added to the cluster tubes. Cells were pelleted in the cluster tubes by centrifuging at 2,000 rpm for 2 min and the supernatant discarded. Cells were then resuspended in 80 µl FACS buffer. Directly before running cells on an LSR II flow cytometer (BD Biosciences), DAPI was added to the cells to a final concentration of 1 µg/ml, which allowed the separation of live from dead cells. Samples were acquired with a LSR II flow cytometer (BD Biosciences) using FACSDIVA software V 6.2 (BD Biosciences). *P. berghei* infected cells were identified by gating on viability and size, removing doublets

and gating on GFP positive but PE (autofluorescene) negative cells (32).

Statistical Analysis

Comparison of antibody titers and ISI results was performed using a Kruskal-Wallis test, which was followed up by a Dunn's multiple comparison test.

TRA was calculated from SMFA data, as 100 × [1- (mean number of oocysts in test/mean number of oocysts in control)] and 95% confidence intervals (95%CIs) of % inhibition in oocyst density from a single or multiple feeding experiments for each test antibody at each concentration were calculated using a zero inflation negative binomial model, as described previously (31).

ISI was calculated from cytometer acquisitions files (.fcs) using FlowJo.V 9.7.6 (Tree Star). The percentage of sporozoite inhibition was calculated as a reduction in the percentage of infected cells observed in untreated wells (average) compared to the percentage of infected cells observed in the presence of serum. The sensitivity limit of the assay was defined as the mean of the negative control + 1.645 standard deviations, i.e., 95% of observed values in the negative control, assuming a Gausian distribution.

A linear regression model was used to evaluate differences in functional activity among vaccine groups. The $\log_{10}$ transformed ratio of the mean oocyst count in control and test samples for SMFA, or the ratio of the percentage of viable, GFP positive hepatoma cells in control and test sample for ISI, was the dependent variable, and the square root of anti-Pfs25 and anti-CSP AU, respectively, were independent variables in the model. In another linear regression analysis, reciprocal of dilution (in a square root scale) from the original pooled serum to the tested IgG in a feeder was utilized as the independent variable, instead of anti-Pfs25 AU. Since the ratio of anti-Pfs25-specific IgG to entire IgG in a sample is considered to be stable before (i.e., in the original pooled serum) and after (i.e., in the purified IgG) protein G affinity purification, the anti-Pfs25 AU in the original pooled serum and in the purified IgG were used to calculate the dilution factor of each test IgG at each test concentration. Differences within the linear regression analyses were determined using Tukeys HSD.

Statistical tests were performed using Prism 6 (GraphPad Software Inc, United States), JMP11 (SAS Institute Inc, United States) or R (version 3.4.1). $P < 0.05$ were considered significant.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

FB and SB: conceived and planned the study and wrote the manuscript. YL: prepared Pfs25-IMX313 vaccine for immunization. FB: performed mouse experiments. FB, IT, and AM: performed ELISAs. KM and CL: performed SMFAs. AMS: generated parasites for the ISI assay. FB and AJS: conducted the ISI assay. KM and FB: performed statistical analyses of the SMFA and ISI results. All authors read and commented on the manuscript.

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CXCR3+ T Follicular Helper Cells Induced by Co-Administration of RTS,S/AS01B and Viral-Vectored Vaccines Are Associated With Reduced Immunogenicity and Efficacy Against Malaria

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A malaria vaccine strategy targeting multiple lifecycle stages may be required to achieve a high level of efficacy. In two Phase IIa clinical trials, we tested immunogenicity and efficacy of RTS,S/AS01B administered alone, in a staggered regimen with viral-vectored vaccines or co-administered with viral-vectored vaccines. RTS,S/AS01B induces high titers of antibody against sporozoites and viral-vectored vaccines ChAd63 ME-TRAP and MVA ME-TRAP induce potent T cell responses against infected hepatocytes. By combining these two strategies, we aimed to improve efficacy by inducing immune responses targeting multiple parasite antigens. Vaccination with RTS,S/AS01B alone or in a staggered regimen with viral vectors produced strong immune responses and demonstrated high levels of protection against controlled human malaria infection. However, concomitant administration of these vaccines significantly reduced humoral immunogenicity and protective efficacy. Strong Th1-biased cytokine responses induced by MVA ME-TRAP were associated with a skew in circulating T follicular helper cells toward a CXCR3+ phenotype and a reduction in antibody quantity and quality. This study illustrates that while a multistage-targeting vaccine strategy could provide high-level efficacy, the regimen design will require careful optimization.

Keywords: malaria, vaccine, T follicular helper cell, antibody, efficacy, CXCR3, RTSS viral-vectored vaccines

INTRODUCTION

Despite years of remarkable success in reducing malaria morbidity and mortality, progress appears to have stalled with 216 million new cases in 2016, 5 million more than 2015 (1). An efficacious vaccine could be an essential tool to enable any further reduction in morbidity and mortality, and for the ultimate goal of eradication (2). The most advanced vaccine candidate, RTS,S, has shown significant short-term protective efficacy and has completed testing in a large Phase III trial (3–7).

Abbreviations: ChAd, chimpanzee adenovirus; CHMI, controlled human malaria infection; CSP, circumsporozoite protein; cTfh, circulating T follicular helper cell; GC, germinal center; ISI, inhibition of sporozoite invasion; ME, multi-epitope; MVA, modified vaccinia virus Ankara; PBMCs, peripheral blood mononucleocytes; pfu, plaque-forming units; TRAP, thrombospondin-related adhesive protein; VE, vaccine efficacy; VP, viral particles.

However, there remains a need to improve efficacy to achieve the goals laid out in the Malaria Vaccine Technology Roadmap (8). To improve efficacy, it may be necessary to develop a vaccine regimen targeting multiple stages of the parasite lifecycle (9). In addition to RTS,S, which targets the pre-liver stage, vaccines are being developed to target liver- and blood-stage parasites, or block parasite transmission and these could be combined into a multistage malaria vaccine program (9–12). It is likely that for a multistage vaccine regimen to provide high-level efficacy, it will need to induce both potent T cell and antibody responses (9).

The primary mechanism by which RTS,S induces protection appears to be antibody responses against the NANP repeat region of the circumsporozoite protein (CSP) on the sporozoite surface (13–16). The viral-vectored vaccines used in this study were chimpanzee adenovirus serotype 63 (ChAd63) and modified vaccinia virus Ankara (MVA), both expressing a multi-epitope (ME) string fused to the *Plasmodium falciparum* protein thrombospondin-related adhesive protein (TRAP). The ME string contains 17 epitopes from potentially protective *P. falciparum* and bacille Calmette–Guérin antigens in addition to epitopes from tetanus toxoid. TRAP is expressed on the surface of sporozoites and contains a thrombospondin domain that binds to heparin sulfate proteoglycans to facilitate sporozoite entry into host hepatocytes. ChAd63 ME-TRAP and MVA ME-TRAP provide protection by inducing $CD8^+$ T cell responses against infected hepatocytes (17). When RTS,S/AS01B and viral-vectored vaccines were tested in a combination regimen, efficacy against controlled human malaria infection (CHMI) was higher for volunteers receiving the combination of vaccines (14/17 subjects protected; vaccine efficacy (VE) 82.4% [95% CI 64–100]) than for those receiving RTS,S/AS01B alone (12/16 subjects protected; VE 75% [95% CI 54–96]), suggesting that TRAP-specific T cell responses could add to the protective effect of RTS,S-induced antibody responses (18). In that study, NANP IgG titers in the combination group were comparable to those in the group given RTS,S/AS01B alone and were significantly higher in protected individuals. Titers of IgG against NANP were negatively correlated with parasitemia at day 7.5, indicating a reduced liver to blood inoculum. However, as viral-vectored vaccinations were given at staggered time points, a minimum of 2 weeks after RTS,S/AS01B, this regimen required five separate clinic visits over a period of 10 weeks. For a vaccine regimen to be logistically and economically feasible for deployment in malaria-endemic regions, the number of clinic visits should be reduced. For this reason, we conducted a further Phase I/IIa clinical trial to assess concomitant administration of RTS,S/AS01B with viral-vectored vaccines (Rampling et al. manuscript under review). On the basis of high efficacy in two previous trials, additional groups were included to test a reduced third dose of RTS,S/AS01B (1/5th, 10 μg) (14, 19). In this study, co-administration of these two vaccine platforms resulted in a significant reduction in humoral immunogenicity and efficacy with only 11/19 volunteers protected (VE 57.9% 95% CI [33.2–76.3]), compared with 14/17 (82.4% 95% CI [54.7–93.9]) in the group receiving RTS,S/AS01B alone.

Durable, high-affinity IgG is generated in germinal center (GC) reactions in secondary lymphoid organs, during which B cells undergo class-switching, somatic hypermutation, and differentiation into memory B cells and plasma cells. T follicular helper cells (Tfh) expressing CD4 and CXCR5 and the transcription factor Bcl-6 provide essential help to B cells for this process in the form of cytokine production (IL-21) and the expression of costimulatory molecules (CD40L, ICOS) (20, 21). Circulating $PD1^+CXCR5^+CD45RA^-$ $CD4^+$ T cells appear to be a peripheral counterpart of conventional lymphoid resident Tfh, may represent GC responses, and are a useful tool for clinical trials in which lymphoid tissue is rarely available for analysis (22–24). Circulating T follicular helper cells (cTfh) can be further defined by differential expression of chemokine receptors CXCR3 and CCR6: Th17-like (cTfh17) $CXCR3^-CCR6^+$, double-positive $CXCR3^+CCR6^+$, Th1-like (cTfh1) $CXCR3^+CCR6^-$, and Th2-like (cTfh2) $CXCR3^-CCR6^-$. These subsets have been associated with varying degrees of helper activity in different contexts (25). In particular, $CXCR3^-$ Tfh have been associated with the production of broadly neutralizing antibodies against HIV and Tfh17 induced by rVSV-ZEBOV vaccination were associated with antibody responses against Ebola (26, 27). Therefore, the type of cTfh induced by vaccination may be an indicator of the quality of the GC reaction and the resulting antibodies produced.

To investigate the mechanisms underlying the reduction in humoral immunogenicity after co-administration of ChAd63-MVA ME-TRAP and RTS,S, we conducted a thorough analysis of the differences in antibody quality and cTfh responses in volunteers receiving RTS,S/AS01B alone (R), RTS,S/AS01B given with viral vectors in a staggered regimen (R2V) or co-administered (R + V). Trial regimens are summarized in **Table 1**. This is the first study to assess the impact of vaccine co-administration on the cTfh response in humans and also defines a functional antibody quality that may explain the improved efficacy observed in RTS,S regimens with a reduced third dose.

MATERIALS AND METHODS

Samples and Study Details

Full details of these studies are available in the clinical trial reports [(18), Rampling et al. manuscript under review]. Healthy adult volunteers were recruited and vaccinated at four UK sites, in Oxford, Southampton, London, and Surrey. The CHMI procedure was performed as previously described using five infectious bites from *P. falciparum* 3D7-strain infected *Anopheles stephensi* mosquitoes at Imperial College, London (28). All subjects were infected with a single batch of infected mosquitoes for each trial, supplied by the Department of Entomology, Walter Reed Army Institute of Research, Washington DC, USA. All vaccinations were administered intramuscularly into the deltoid region of the arm. For participants who received concomitant vaccinations, RTS,S/AS01B was administered first followed by the viral-vectored vaccine in the same site no longer than 5 min later.

Ethics Statement

All volunteers gave written informed consent prior to participation, and the studies were conducted according to the principles of the Declaration of Helsinki and in accordance with Good

TABLE 1 | Vaccination schedules.

	Co-administration study (VAC59)					Staggered study (VAC55)		
	RTS,S/AS01B (R)		RTS,S/AS01B co-administered with viral vectors (R + V)			RTS,S/AS01B and viral vectors staggered (R2V)	RTS,S/AS01B (R)	
Group	G1 R-R-R	G2 R-R-r	G3 RA-RM-RM	G4 RA-RM-rM	Controls	R-A-R-R-M	R-R-R	Controls
No. volunteers enrolled	10	10	10	11	4	20	17	6
No. volunteers at C-1	9	10	10	9	4	17	16	6
No. volunteers challenged	8	9	10	9	4	17	16	6
Week 0	R	R	RA	RA		R	R	
Week 2						A		
Week 4	R	R	RM	RM		R	R	
Week 8	R	r	RM	rM		R	R	
Week 10						M		
Week 11	CHMI	CHMI	CHMI	CHMI	CHMI			
Week 12						CHMI	CHMI	CHMI
Efficacy: sterilely protected volunteers	6/8 (75%)	8/9 (89%)	6/10 (60%)	5/9 (56%)	0/4	(14/17) 82%	(12/16) 75%	0/6

R, 50 μg RTS,S/AS01B; r, 10 μg RTS,S/AS01B; A, 5 × 10^{10} viral particles (vp) ChAd63 ME-TRAP; M, 2 × 10^{8} plaque-forming units (pfu) MVA ME-TRAP; CHMI, controlled human malaria infection; (R), RTS,S/AS01B groups; (R2V), RTS,S/AS01B and viral-vectored vaccinations staggered by 2 weeks; (R + V), RTS,S/AS01B and viral vectors co-administered.

Clinical Practice. The trials were registered with ClinicalTrials.gov (Ref: NCT01883609 and NCT02252640). The study protocols were approved by the UK National Research Ethics Service, Committee South Central—Oxford A (Refs: 13/SC/0208 and 14/SC/0227), the Western Institution Review Board (Ref: 20130698), and the UK Medicines and Healthcare products Regulatory Agency (Refs: 21584/0317/001-0001 and 21584/0333/001-0001). The Local Safety Committee provided safety oversight for both trials and GCP compliance was monitored by the Clinical Trials and Research Governance Team (CTRG) of the University of Oxford.

Total IgG ELISA

ELISA 96-well plates were coated with a synthetic peptide (Eurogentec, Liège, Belgium) based on the PfCSP repeat region ($(NANP)_6C$) diluted to 0.2 μg/mL in 100 μL dPBS per well and incubated overnight at room temperature (RT). Plates were washed six times with PBS containing 0.5% Tween-20 (PBS/T) and blocked with casein for 1 h at RT. Plates were washed again and serum samples diluted in casein at 1:100, 1:500, 1:1,000, or 1:5,000, were added for 2 h at RT. After washing again, secondary antibody (goat anti-human IgG conjugated to alkaline phosphatase, Sigma) was added at 1:1,000 in casein for 1 h at RT. Plates were washed a final time and developed using 4-nitrophenyl phosphate in diethanolamine buffer (Pierce, Rockford, IL, USA). Optical density (OD) was read at 405 nm using an ELx800 microplate reader (Bio-Rad, Hercules, CA, USA). A reference pool of positive serum formed a standard curve on each plate and was used to calculate ELISA units for each sample. An internal control was included on each plate to standardize between assays. All samples were tested in triplicate.

Isotype ELISA

Isotype ELISAs were conducted as described above, except that all serum samples were diluted to 1:100 and added to the plate in duplicate wells on each of six plates. One of six secondary antibodies against IgG1, IgG2, IgG3, IgG4, IgM, or IgA was added to each plate at 1:1,000 in casein before developing as above. Blank wells and internal development controls were included on each plate. A "seropositive cut-off" value was calculated for each isotype or subclass using the mean plus 3 SDs of 36 UK malaria-naïve serum samples.

Indirect Immunofluorescence Assay (IFA)

Chambered microscope slides coated with *P. falciparum* sporozoites were stored at −80°C until use. Slides were brought to RT and then fixed for 15 min in 4% paraformaldehyde. After washing twice in PBS for 5 min, slides were blocked for 1 h in casein. Slides were washed as before and 10 μL of serum sample diluted 1:100 in casein was added to each well. Slides were incubated for 30 min at RT in a humidity chamber then wells were individually washed with PBS three times for 5 min. Secondary antibody (anti-IgG-AlexaFluor488) was diluted 1:800 in casein and 15 μL was added to each well for 30–45 min in a humidity chamber at RT protected from the light. Slides were washed a final time, rinsed in distilled water and left to dry before mounting with DAPI-containing media. Slides were left to set overnight at 4°C before being examined under a Leica DMI3000 B microscope. Images were captured in QCapturePro software using brightfield illumination, GFP and DAPI filters at set exposure levels. ImageJ software was used to measure the median fluorescence intensity (MFI) for five sporozoites in each well and an average was taken.

Inhibition of Sporozoite Invasion (ISI) Assay

The ISI assay was carried out as previously described (29). Human hepatoma cells (HC04) cultured in R10 medium (RPMI 1640 with 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine) were added to 96-well culture plates at 30,000 cells/well and left to settle overnight at 37°C, 5% CO_2. Viable GFP-labeled *Plasmodium berghei* sporozoites expressing *P. falciparum* CSP at the *P. berghei* CSP locus (*P. berghei* PfCSP@CSP) were obtained by dissecting infected *A. stephensi* mosquitoes. Salivary

glands were pooled into RPMI 1640 medium and homogenized. Sporozoites were counted and diluted to 100,000/mL in RPMI 1640. Culture medium was aspirated from the hepatoma cells then 100 µL of serum diluted 1:5 in R10 and 100 µL of sporozoite dilution (10,000 sporozoites, 10% final serum concentration) were added to each well. Samples were tested in duplicate and an average calculated. "Hepatoma only" wells and infectivity control wells that contained hepatoma cells and sporozoites but no serum were included. Pre-vaccination and C-1 samples were run for each volunteer. After incubation for 20–26 h at 37°C, medium was aspirated, and plates were washed with 90 µL/well dPBS. Cells were trypsinized, re-suspended in 65 µL dPBS with 1% bovine serum albumin (BSA), and acquired immediately using a BD LSRII. DAPI stain was added to each sample just before acquisition. Data were analyzed in Flow Jo software v10.6 (Tree Star Inc., Ashland, OR, USA) according to a predefined gating strategy. The percentage of sporozoite inhibition was calculated for each sample (average of duplicate wells) based on the reduction in the percentage of infected cells compared with the infectivity controls (average of 4–6 wells).

Ex Vivo IFNγ ELISpot

Ex vivo ELISpot assays were performed for TRAP-specific T cell responses as previously described (10). Average responses were taken across triplicate wells, background subtracted and then responses in individual pools were summed.

cTfh Phenotyping and ICS

Surface phenotyping of cTfh was carried out using cryopreserved peripheral blood mononucleocytes (PBMCs). Thawing was performed rapidly in a water bath and cells were rested for 2 h at 37°C with 5% CO_2 and Benzonase at 25U/10^6 PBMC (Novagen, Madison, WI, USA) before staining. For surface phenotyping 1–2 million PBMC were stained. Cells were washed in FACS buffer (PBS containing 0.1% BSA and 0.01% sodium azide) and stained with LIVE/DEAD aqua amine reactive dye (Life Technologies Ltd., Carlsbad, CA, USA) for 20 min at RT in the dark. Cells were washed in FACS buffer and a cocktail of antibodies for Tfh surface staining (**Table 2**) was added for 30 min at RT. Cells were washed again in FACS Buffer and re-suspended in PBS containing 1% paraformaldehyde, prior to acquisition on a BD LSR II using FACSDiva v6.2 (BD Biosciences, Franklin Lakes, NJ, USA) on the day of staining. Compensation control beads (OneComp Beads, eBioscience, San Diego, CA, USA, ArC Amine Reactive Beads, Invitrogen, Carlsbad, CA, USA) were stained according to the manufacturer's instructions for compensation between parameters. A median of 100,000 live $CD4^+$ cells were acquired [inter-quartile range (IQR) 25% = 68,406, 75% = 143,000] per sample. Data analysis was performed using Flow Jo v9.6.2 (Tree Star Inc.). IFNγ production was measured after overnight stimulation with 2 µg/mL of a pool of 31 peptides spanning the CSP antigen (15mers overlapping by 11 amino acids, at 2 µg/mL, all volunteers) or 10 µg/mL superantigen *Staphylococcus* enterotoxin B (SEB). Brefeldin A and monensin were added at 10 µg/mL after 2 h. The staining protocol was the same as for cTfh phenotyping, except that after surface staining, cells were permeabilized with fix/perm buffer (BD biosciences) then stained intracellularly at RT for 30 min with IFNγ-FITC (1:250, eBioscience), washed and fixed in 1% paraformaldehyde. Acquisition and analysis was performed as for cTfh phenotyping.

TABLE 2 | Circulating T follicular helper cell phenotyping panel.

Marker	Fluorophore	Supplier	Clone	Dilution	Volume (µL)
CD3	Alexa Fluor 700	eBioscience	UCHT1	1:33	1.5
CD4	APC-eFluor 780	eBioscience	SK3 (SK-3)	1:50	1
CD45RA	eFluor450	BioLegend	HI101	1:50	1
CXCR5	PerCP-eFluor 710	BioLegend	MU5UBEE	1:16	3
CXCR3	APC	BioLegend	1C6/CXCR3	1:16	3
CCR6	PE	BioLegend	G034E3	1:16	3
PD-1	BV650	BioLegend	EH12.2H7	1:100	0.5
Live/dead	AmCyan	Invitrogen	N/A	1:500	0.1
IFNγ (in ICS assay)	FITC	eBioscience	4S.B3	1:250	0.2

Multiplex Cytokine Assay

Between 1 and 2 million PBMC from the C-1 time point were plated per well in a 96-well plate and stimulated for 21 h at 37°C. Cells were stimulated either with a pool of 31 peptides spanning the CSP antigen (15mers overlapping by 11 amino acids, at 2 µg/mL, all volunteers) or 10^6 pfu of MVA. Supernatants were taken and stored at −20°C in 96-well U-bottom polypropylene plates until use. Cytokine concentrations in the supernatants were measured using the LEGENDplex human Th cytokine panel 13-plex assay (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. The samples were read on the same day on a BD LSR II using FACSDiva v6.2 (BD Biosciences) with 5,000 beads acquired per sample. Data analysis was conducted using the LEGENDplex data analysis software.

Statistical Analysis

Data tested negative for a normal distribution by D'Agostina–Pearson omnibus normality test; therefore, non-parametric tests were used and medians with IQRs are presented. Mann–Whitney analysis was used to compare differences between two groups. Kruskal–Wallis with Dunn's multiple comparisons post-test was used to compare responses across multiple groups at a given time point. Wilcoxon matched-pairs analysis was used to compare responses at two time points within a group. Spearman's rank was calculated for correlations. $P < 0.05$ was considered significant and all P values are two-tailed. Analyses were performed in GraphPad Prism, version 7.

RESULTS

Reduced Quantity and Quality of Antibody Responses When RTS,S/AS01B Is Co-Administered With Viral-Vectored Vaccines

Antibody responses against the NANP repeat region of CSP were measured in each of the clinical trials and reported separately (18) (Rampling et al. manuscript under review). Total

NANP-specific IgG was measured by ELISA at baseline (day 0, D0), D28, D42, D56, D76 (the day before CHMI, C-1, in the co-administration trial) and 35 and 90 days after CHMI (C + 35, C + 90). In the staggered regimen trial, anti-NANP-specific IgG was also measured at D83 (C-1 for that trial) as the CHMI was 1 week later to accommodate the additional vaccinations (**Figure 1A**). Titers were comparable in all regimens after the first two vaccinations but failed to re-boost after the third vaccination

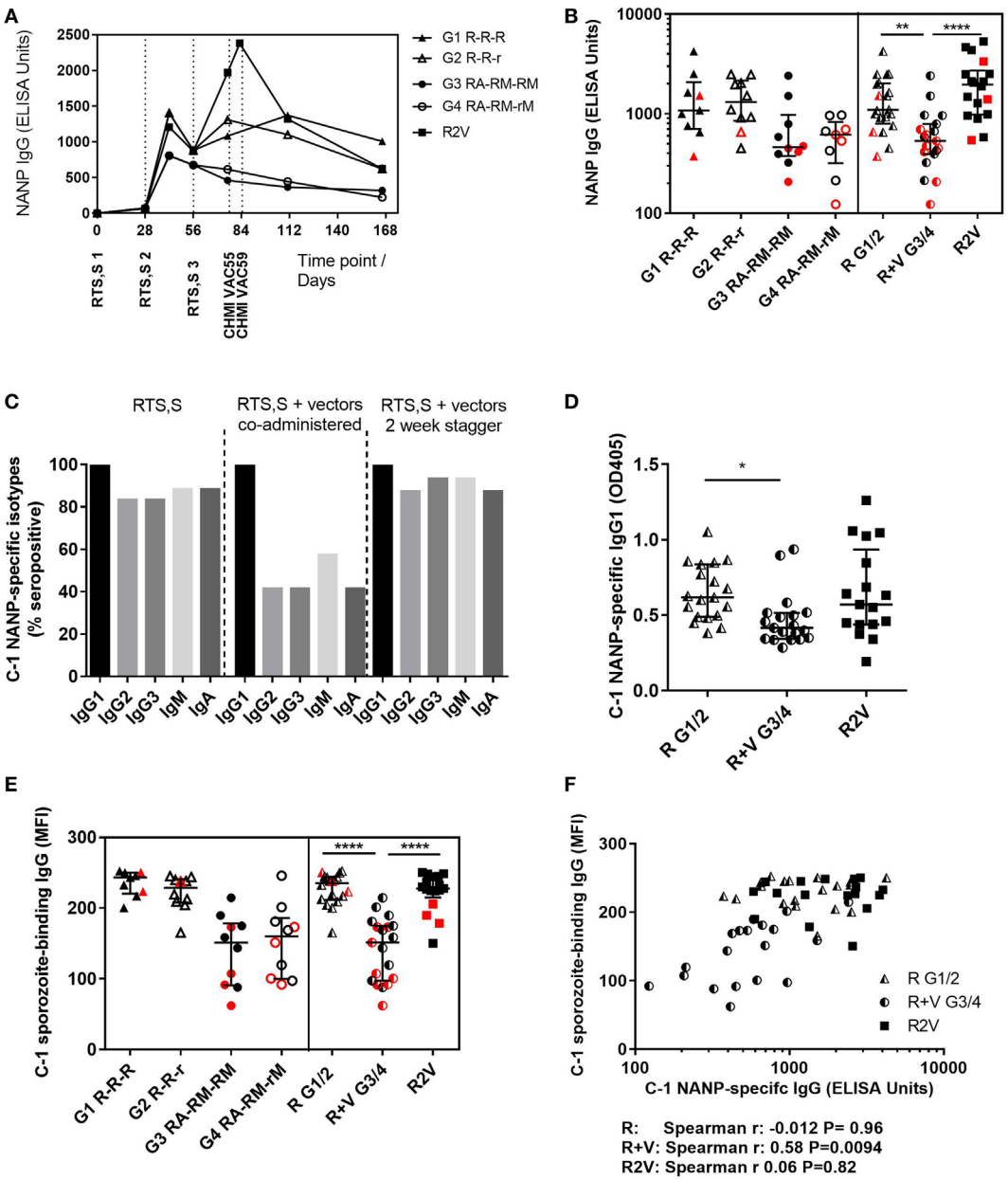


FIGURE 1 | Antibody quantity and quality. **(A)** Median NANP-specific IgG time courses. **(B)** Total IgG titers against the CSP repeat region NANP in each group at D76 (2 weeks after third dose of RTS,S/AS01B), in G1/2 combined (R) and G3/4 combined (R + V) or in the staggered regimen (R2V), Kruskal–Wallis with Dunn's post-test $P < 0.0001$. **(C)** NANP-specific isotype and IgG subclass responses at C-1. **(D)** NANP-specific IgG1 at C-1, Kruskal–Wallis $P = 0.0062$. **(E)** MFI of sporozoite-binding IgG at C-1 measured by IFA, Kruskal–Wallis $P < 0.0001$. **(F)** Relationship between NANP IgG titer and level of sporozoite-binding at C-1. Volunteers receiving RTS,S/AS01B alone (G1/2, R) Spearman r: −0.012, $P = 0.96$, RTS,S/AS01B co-administered with vectors (G3/4, R + V) Spearman r: 0.58, $P = 0.0094$ or RTS,S/AS01B and viral vectors given in a staggered regimen Spearman r: 0.06, $P = 0.82$. Medians + IQRs shown and non-protected volunteers highlighted in red for all column graphs. Abbreviations: R G1/2, RTS,S/AS01B vaccinated; R + V G3/4, RTS,S/AS01B and viral-vectored vaccines co-administered; R2V, RTS,S/AS01B and viral-vectored vaccines at a 2-week stagger; A, ChAd63 ME-TRAP; M, MVA ME-TRAP; R, 50 μg third dose of RTS,S/AS01B; r, 10 μg third dose of RTS,S/AS01B; CHMI, controlled human malaria infection; C-1, day before CHMI; CSP, circumsporozoite protein; TRAP, thrombospondin-related adhesive protein; IQRs, inter-quartile ranges; IFA, immunofluorescence assay; MFI, median fluorescence intensity.

in the co-administration regimen, resulting in significantly lower titers in these groups at D76 (**Figure 1B**, median ELISA units, R: 1,102 IQR [757–2,035], R + V: 533 IQR [394–790], R2V: 1,969 IQR [983–2,724] Kruskal–Wallis $P < 0.0001$). There were no significant differences in NANP IgG titers between groups receiving a full or reduced third dose of RTS,S/AS01B either alone or co-administered with viral-vectored vaccines. The highest titers were seen in the staggered regimen at D83 (4 weeks after the third dose of RTS,S/AS01B), although there was no comparable time point in the co-administration study.

Isotype and subclass responses were measured by ELISA against the NANP repeat region at C-1 in both trials (**Figure 1C**). Titers were measured for IgG1–4, IgM, and IgA. No NANP-specific IgG4 was detected in any volunteers (data not shown). Over 80% of volunteers given RTS,S/AS01B alone were seropositive for NANP-specific IgG2, IgG3, IgM, and IgA. There were significant reductions in seroconversion for these isotypes/subclasses in groups that received concomitant viral-vectored vaccinations, but not in the staggered regimen. All volunteers were positive for NANP IgG1 and titers were comparable in the RTS,S/AS01B only groups (R) and the staggered administration group (R2V), but were significantly reduced in the co-administration regimen (R + V) (**Figure 1D**, median OD, R: 0.617 IQR [0.488–0.835], R + V: 0.415 [0.343–0.514], R2V: 0.570 [0.438–0.935] Kruskal–Wallis $P = 0.0062$). There were no significant differences in IgG1 titers between groups receiving full or reduced third doses of RTS,S/AS01B (G1 R-R-R vs G2 R-R-r and G3 RA-RM-RM vs G4 RA-RM-rM, data not shown).

Antibody binding to fixed whole sporozoites was measured for all volunteers at C-1 using an indirect IFA. Sporozoite-binding IgG was significantly lower in the co-administration regimen but the staggered regimen was comparable to RTS,S/AS01B alone (**Figure 1E**, MFI, R: 238 IQR [213–245] R + V: 151 [98–175], R2V: 228 [215–245] Kruskal–Wallis $P < 0.0001$). There were no differences between groups receiving full or reduced third doses of RTS,S/AS01B. Sporozoite-binding was significantly associated with NANP IgG titers in the co-administration groups (**Figure 1F**, Spearman r: 0.58, $P = 0.0094$). There was no association in the RTS,S/AS01B alone or staggered regimens (Spearman r: −0.012, $P = 0.96$ and r: 0.058, $P = 0.83$, respectively), in which sporozoite-binding was higher for a given NANP IgG titer than in the co-administration regimen.

ISI Is Associated With Protection From CHMI

The functional quality of vaccine-induced antibodies was assessed using an *in vitro* assay measuring the ability of serum to block sporozoite infection of hepatoma cells (29). A defined gating strategy was used to identify infected hepatoma cells (**Figure 2**).

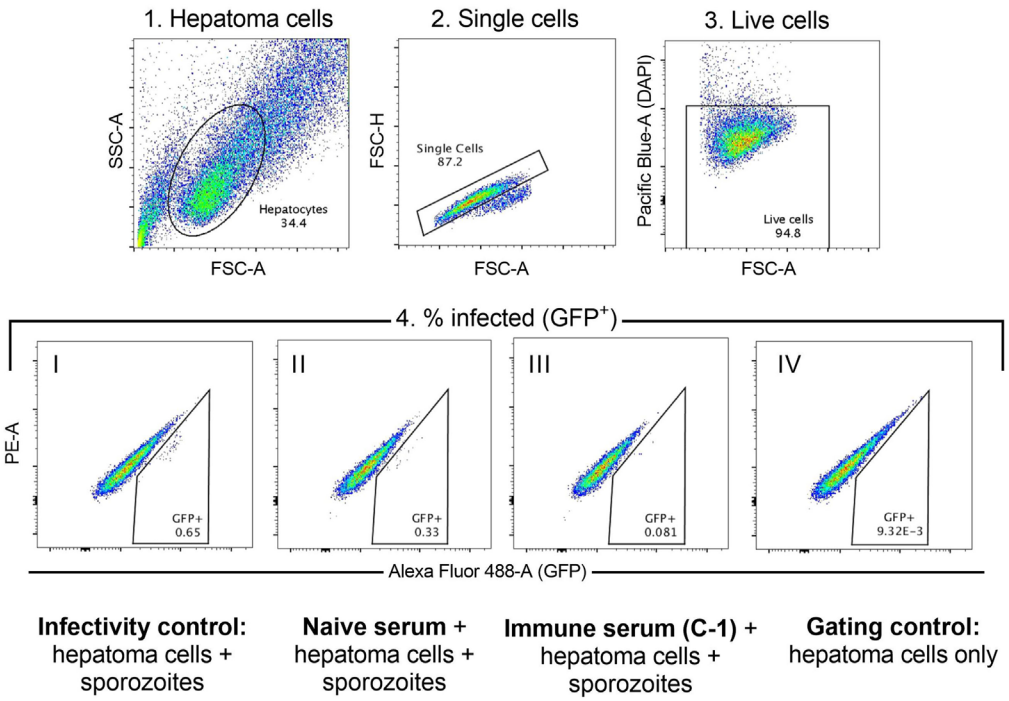


FIGURE 2 | Inhibition of sporozoite invasion assay. Hierarchical gating strategy used to determine the percentage of hepatoma cells infected with GFP-expressing sporozoites. 1. Hepatoma cells are gated based on size to exclude debris and large cell clumps. 2. Singlets are gated to exclude smaller cell clumps. 3. Live cells (DAPI-negative) are gated to exclude dead cells. 4. Cells infected with the GFP-expressing sporozoites are GFP$^+$. Cells are gated against both GFP (Alexa Fluor-AF488 channel, GFP$^+$) and the adjacent channel (PE$^-$) to exclude signal that is caused by autofluorescence. Representative populations in each of the conditions are shown: I. Infectivity control—sporozoite infection of hepatoma cells in the absence of any serum; II. Sporozoite infection of hepatoma cells in the presence of naïve serum (D0); III. Sporozoite infection of hepatoma cells in the presence of immune serum (C-1); IV. Gating control—hepatoma cells only, no sporozoites or serum added.

The percentage of infection blocked by vaccine-induced antibody was significantly lower in the co-administration regimen compared with RTS,S/AS01B alone (**Figure 3A**, median percentage infection blocked R, 90% IQR [76–98], R + V 80% [52–89], Mann–Whitney $P = 0.016$). However, blocking ability was significantly higher in groups receiving a reduced third dose of RTS,S/AS01B than those receiving three standard doses, even when this dose was co-administered with viral-vectored vaccines (**Figure 3A**, medians + IQRs G1 R-R-R: 80% [71.5–91.5], G2 R-R-r: 96% [89.3–98], G3 RA-RM-RM: 55% [50–73.5], G4 RA-RM-rM: 89% [85–95], Mann–Whitney analyses G1 R-R-R vs G2 R-R-r $P = 0.014$, G3 RA-RM-RM vs G4 RA-RM-rM $P < 0.0001$, G1 R-R-R and G3 RA-RM-RM vs G2 R-R-r and G4 RA-RM-rM $P < 0.0001$). ISI was significantly associated with C-1 NANP IgG titer for groups receiving three standard doses of RTS,S/AS01B (**Figure 3B**, G1 R-R-R and G3

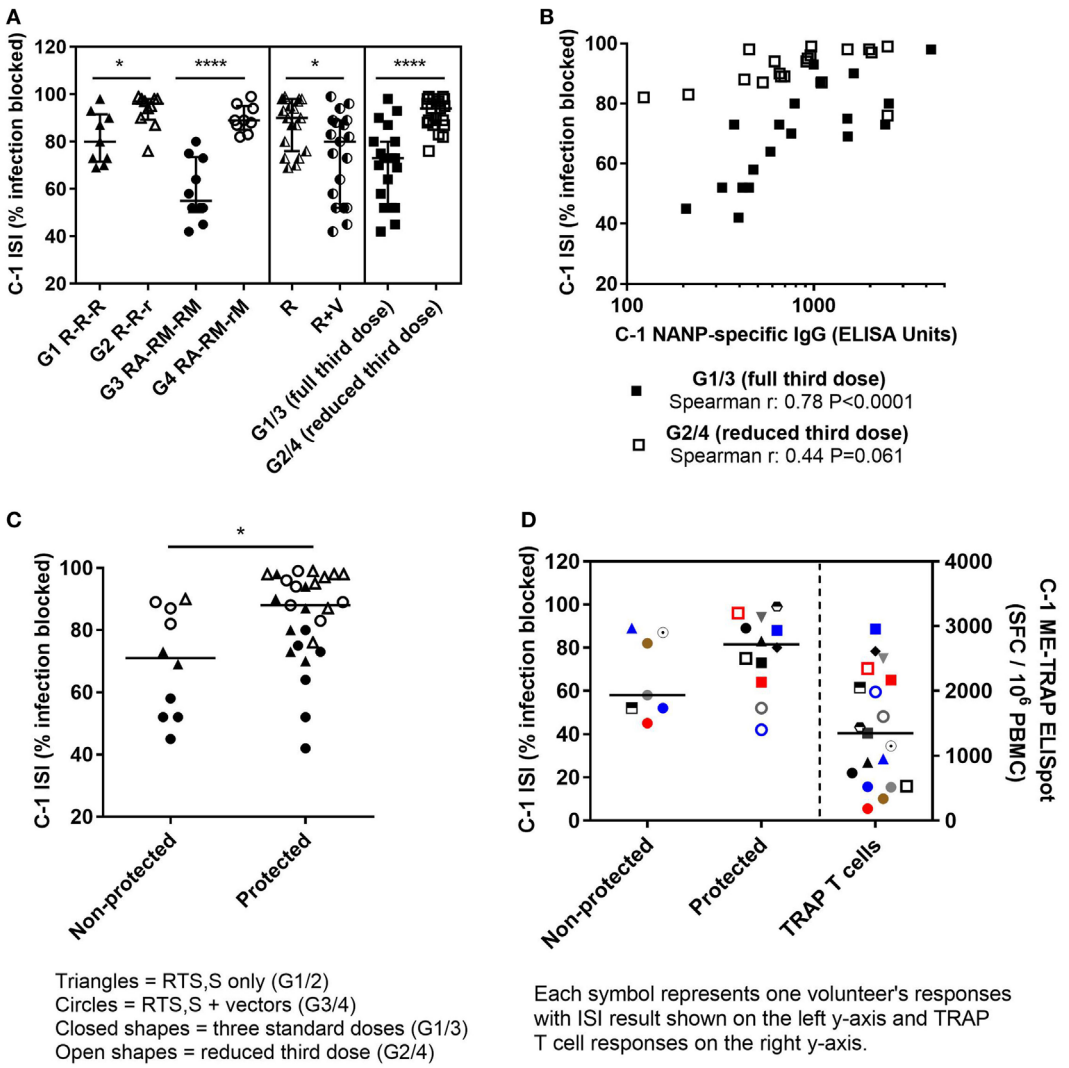


FIGURE 3 | ISI results. **(A)** Percentage of sporozoite invasion into hepatoma cells that was blocked by serum at C-1. Comparison of groups given three standard doses of RTS,S/AS01B or a reduced (1/5th) third dose of RTS,S/AS01B. Mann–Whitney analyses G1 R-R-R vs G2 R-R-r $P = 0.014$, G3 RA-RM-RM vs G4 RA-RM-rM $P < 0.0001$, G1/2 (R) vs G3/4 (R + V) $P = 0.016$, G1/3 (full third dose of RTS,S/AS01B) vs G2/4 (reduced third dose of RTS,S/AS01B) $P < 0.0001$, medians + IQRs. **(B)** Relationship between C-1 NANP IgG titers and sporozoite-blocking ability in individuals who received three full doses of RTS,S/AS01B with or without vectored vaccines (G1 and G3, closed squares, Spearman *r*: 0.78, $P < 0.0001$) or a reduced third dose of RTS,S/AS01B with, or without vectored vaccines (G2 and G4, open squares, Spearman *r*: 0.44, $P = 0.061$). **(C)** Percentage of sporozoite invasion blocked by C-1 serum protected and non-protected individuals, Mann–Whitney $P = 0.017$. Closed triangles: G1, open triangles: G2, closed circles: G3, open circles: G4. **(D)** Percentage of sporozoite invasion blocked by serum at C-1 in protected and non-protected volunteers in G3/4, R + V (left *Y*-axis) and corresponding TRAP T cell responses (right *Y*-axis) at C-1 for each volunteer measured by IFNγ ELISpot, summed responses from T9/96 TRAP pools + ME, spot-forming cells per million PBMC (SFC/10^6 PBMC), lines at medians. Abbreviations: R G1/2, RTS,S/AS01B vaccinated; R + V G3/4, RTS,S/AS01B and viral-vectored vaccines co-administered; R2V, RTS,S/AS01B and viral-vectored vaccines at a 2-week stagger; A, ChAd63 ME-TRAP; M, MVA ME-TRAP; R, 50 μg third dose of RTS,S/AS01B; r, 10 μg third dose of RTS,S/AS01B; CHMI, controlled human malaria infection; C-1: day before CHMI; ISI, inhibition of sporozoite invasion; PBMCs, peripheral blood mononucleocytes; TRAP, thrombospondin-related adhesive protein; IQRs, inter-quartile ranges.

RA-RM-RM Spearman r: 0.78, $P < 0.0001$) but not in groups receiving a reduced third dose, in which blocking ability was higher even at lower NANP titers (G2 R-R-r and G4 RA-RM-rM Spearman r: 0.44, $P = 0.061$). The relationship between blocking ability and the NANP-specific isotype/subclass titers was also assessed (Figure S1 in Supplementary Material). Relationships between all subclasses and isotypes tested and blocking ability demonstrated a similar pattern to total IgG, with a positive correlation in G1/3 and high blocking regardless of titer in G2/4. However, IgG1 was the only isotype that showed an association with blocking ability in the data set as a whole (G1–4, Spearman r: 0.42, $P = 0.009$).

Blocking ability was associated with protection from malaria after CHMI, with significantly higher percentages of infection blocked in protected than non-protected volunteers (**Figure 3C**, medians + IQRs protected: 88% [75–97], non-protected: 71% [52–87.5] Mann–Whitney $P = 0.019$). TRAP-specific T cell responses elicited by the viral-vectored vaccines were measured by IFNγ ELISpot and previously reported (Rampling et al. manuscript under review). T cell responses (IFNγ responses against summed T9/96 TRAP pools + the multi-epitope, ME) were not reduced by co-administration and responses were significantly higher in protected than non-protected individuals in these groups (Rampling et al. manuscript under review). Volunteers in the co-administration groups that were protected despite having low levels of sporozoite-blocking antibody had high TRAP-specific T cell responses (**Figure 3D**).

Proportion of CXCR3$^+$ cTfh Increases When RTS,S/AS01B Is Co-Administered With Viral-Vectored Vaccines and Negatively Correlates With Antibody Responses

To determine whether cellular differences associated with the reduction in antibody responses in the co-administration regimen could be detected in the blood, cTfh were phenotyped at C-1 by surface staining and flow cytometry using a defined gating strategy (**Figure 4A**). Total cTfh were analyzed for all volunteers in the co-administration study (except 1 volunteer in G4 for which there were no cryopreserved cells remaining) and 10 volunteers in the staggered administration study with enough cryopreserved cells remaining for the experiment (**Figure 4B**). The proportion of cTfh (PD1$^+$CXCR5$^+$) within memory CD4$^+$ T cells ranged from 0.1 to 4.8%, was comparable across groups and did not correlate with CSP- or TRAP-specific antibody responses. Subsets within cTfh were identified using CXCR3 and CCR6: cTfh17 (CXCR3$^-$CCR6$^+$), CXCR3$^+$ [including double-positive (CXCR3$^+$CCR6$^+$) and cTfh1 (CXCR3$^+$CCR6$^-$)] and cTfh2 (CXCR3$^-$CCR6$^-$). Volunteers who received RTS,S/AS01B co-administered with viral-vectored vaccines had significantly higher frequencies of CXCR3$^+$ cTfh and significantly lower frequencies of cTfh2 than those who received RTS,S/AS01B alone, while the staggered administration group had an intermediate phenotype which was not significantly different to either of the other regimens (**Figure 4C**). There were no significant differences in frequencies of any population between G1 R-R-R and G2 R-R-r or G3 RA-RM-RM and G4 RA-RM-rM (data not shown). In the co-administration regimen, the percentage of CXCR3$^+$ cTfh was negatively correlated with antibody responses to both vaccines (**Figure 4D**, NANP Spearman r: −0.78, $P = 0.0001$, TRAP Spearman r: −0.50, $P = 0.036$). For groups that received RTS,S/AS01B alone, the frequency of CXCR3$^+$ cTfh was lower, and there was no association with antibody responses (**Figure 4E**, Spearman r: 0.32, $P = 0.18$). Although in the staggered regimen the proportion of CXCR3$^+$ cTfh was comparable to that in the co-administration regimen, they were not associated with a reduction in antibody responses in this regimen (Spearman r: 0.26, $P = 0.47$). The proportion of CXCR3$^+$ in CXCR5$^-$ memory CD4$^+$ T cells was not associated with antibody responses in any regimen (data not shown).

Co-Administration of Viral-Vectored Vaccines With RTS,S/AS01B Drives Th1-Biased Cytokine Responses Which Are Associated With the Increase in CXCR3$^+$ cTfh and Reduction in Antibody Responses

Concentrations of a range of T-helper cytokines in the supernatant of C-1 PBMC from the co-administration study were measured using a cytometric bead array (LEGENDplex, BioLegend). PBMCs were stimulated with CSP (all groups) or MVA (G3&4) and concentrations of IL-5, IL-13, IL-2, IL-6, IL-9, IL-10, IFNγ, TNFα, IL-17A, IL17-F, IL-4, IL-21, and IL-22 were measured (**Figure 5A**). High concentrations of IL-2, IFNγ, TNFα, IL-6, and IL-22 were detected. The concentration of IFNγ was particularly high in the supernatant from MVA-stimulated PBMCs where all samples produced >3,000 pg/mL. In comparison, IFNγ responses were significantly lower after CSP stimulation, with no detectable IFNγ in 9/34 samples and less than 1,000 pg/mL in most where responses were detected. However, PBMCs from volunteers in the co-administration groups produced more IFNγ in response to CSP stimulation than those from volunteers who received RTS,S/AS01B alone (**Figure 5B**, median pg/mL + IQR, R CSP: 114 [1–348], R + V CSP: 311 [96–610], R + V MVA: 16,796 [11,409–20,462], Kruskal–Wallis $P < 0.0001$). In addition, IFNγ was a greater proportion of the cytokine response to CSP stimulation in PBMC from G3/4 (R + V) volunteers who were not sterilely protected after CHMI than those who were protected (**Figure 5C**).

The concentration of IFNγ in the CSP supernatant was positively associated with the proportion of CXCR3$^+$ cTfh (**Figure 6A**, Spearman r: 0.41, $P = 0.01$) and negatively with the proportion of cTfh17 within cTfh (**Figure 6B**, Spearman r: −0.63, $P < 0.0001$). In addition, there was a negative association between the concentration of IFNγ in the CSP supernatant and the ability of antibody to block sporozoite entry into hepatocytes (**Figure 6C**, Spearman r: −0.79, $P = 0.0001$). Analysis of IFNγ production by ICS of cTfh after stimulation with CSP or the superantigen SEB showed very low frequencies of these cells expressing IFNγ [**Figure 6D**, less than 2% in G1/2 (R) and less than 3% in G3/4 (R + V) after CSP stimulation].

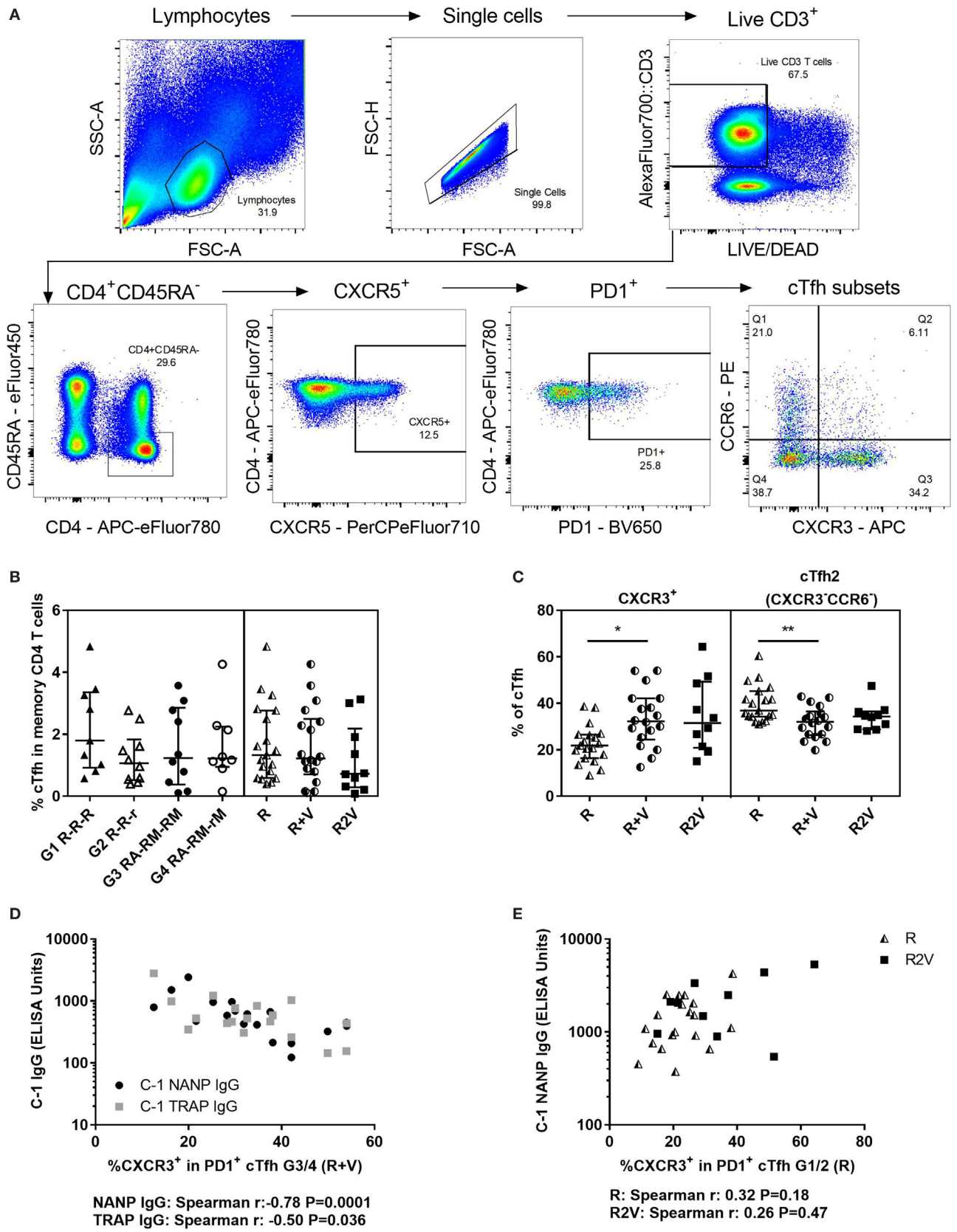


FIGURE 4 | Continued

FIGURE 4 | Total cTfhs. **(A)** Gating strategy for cTfh phenotyping using cell surface staining and flow cytometry. **(B)** Percentage of cTfh ($PD1^+CXCR5^+$) within memory CD4 T cells ($CD45RA^-$) at C-1. **(C)** Subsets within cTfh (at C-1) defined by chemokine receptor expression: cTfh2 ($CXCR3^-CCR6^-$), or $CXCR3^+$; including double-positive, dp ($CXCR3^+CCR6^+$), and cTfh1 ($CXCR3^+CCR6^-$). Kruskal–Wallis analyses; cTfh2 $P = 0.009$, $CXCR3^+$ $P = 0.01$. **(D)** Relationship between percentage of $CXCR3^+$ cTfh and antibody responses at C-1 in individuals who received RTS,S/AS01B co-administered with viral-vectored vaccines (G3/4). NANP IgG (Spearman r: −0.78, $P = 0.0001$), TRAP IgG (Spearman r: −0.50, $P = 0.036$). **(E)** Relationship between percentage of $CXCR3^+$ cTfh and C-1 NANP IgG in individuals vaccinated with RTS,S/AS01B alone (R, G1/2, Spearman r: 0.32, $P = 0.18$) or RTS,S/AS01B and viral-vectored vaccines in a staggered regimen (R2V, Spearman r: 0.26, $P = 0.47$). Abbreviations: R G1/2, RTS,S/AS01B vaccinated; R + V G3/4, RTS,S/AS01B and viral-vectored vaccines co-administered; R2V, RTS,S/AS01B and viral-vectored vaccines at a 2-week stagger; A, ChAd63 ME-TRAP; M, MVA ME-TRAP; R, 50 μg third dose of RTS,S/AS01B; r, 10 μg third dose of RTS,S/AS01B; CHMI, controlled human malaria infection; C-1, day before CHMI; cTfh, circulating T follicular helper cell; TRAP, thrombospondin-related adhesive protein.

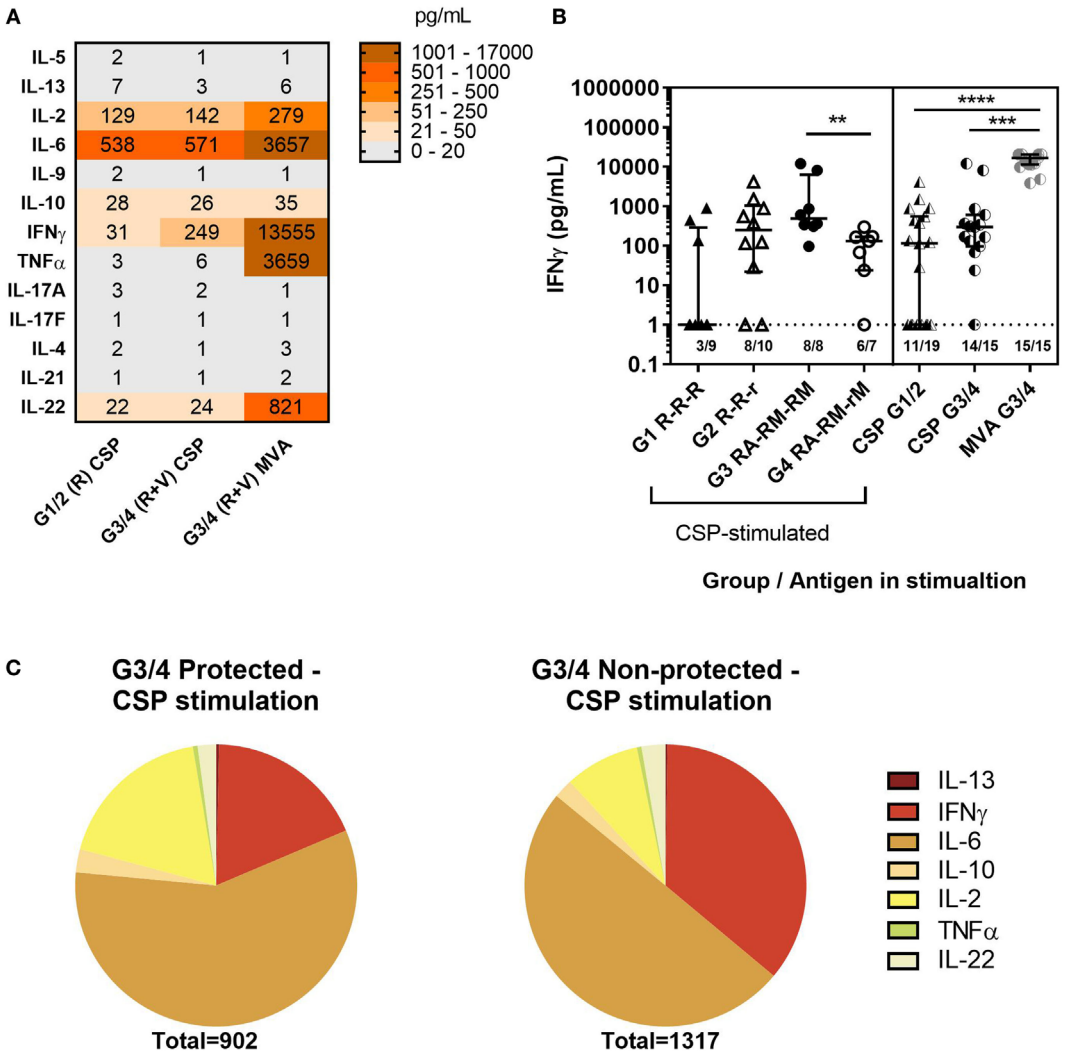
DISCUSSION

Viral-vectored vaccines ChAd63 ME-TRAP and MVA ME-TRAP given with RTS,S/AS01B in a staggered regimen induced high titers of antibodies against sporozoites and potent T cell responses against infected liver cells (18). This regimen required five separate vaccinations and would likely be impractical and uneconomical for deployment in malaria-endemic regions. One way to overcome this obstacle would be to co-administer the RTS,S/AS01B and viral-vectored vaccines. Co-administration of vaccines can lead to interactions between the immune responses, which may be beneficial, enhancing the response as seen after co-administration of the live and attenuated polio vaccines or may result in a reduction of immunogenicity as is the case for multivalent inactivated or live viral vaccines (30, 31).

We examined the immune responses induced by both vaccines when co-administered to assess the extent of the immunological interaction between RTS,S/AS01B and viral-vectored vaccines. An *in vitro* assay measuring the ability of vaccine-induced antibody to block sporozoite invasion of hepatocytes was used to assess the functional quality of antibody. Infection-blocking ability was associated with protection. However, some volunteers who received viral-vectored vaccines were protected despite having antibodies with only low levels of infection-blocking activity. These volunteers had some of the highest TRAP-specific T cell responses, suggesting that cellular responses may provide protection by killing infected hepatocytes in volunteers who do not produce sufficient anti-NANP titers to block sporozoite entry. This demonstrates the potential of a multistage targeting regimen to provide high-level efficacy if each vaccine can be given without interfering with the immunogenicity of the other. However, co-administration of viral-vectored vaccines with RTS,S/AS01B in this study induced a strong Th1 cytokine response and increased proportions of $CXCR3^+$ cTfh, which were associated with reduced antibody quantity and quality and lower efficacy in these groups.

In addition, we observed qualitative differences in RTS,S-induced antibody responses when a reduced third (1/5th, 10 μg) dose was given compared with three standard doses (50 μg). Although NANP IgG titers were comparable, administration of a reduced third dose of RTS,S/AS01B-induced antibodies that were capable of blocking a significantly higher level of sporozoite infection *in vitro*—a measure which was associated with protection from CHMI. Previously, a reduced third dose of RTS,S/AS01B was shown to provide higher levels of protection (14, 19) and in a more recent study, a fractional third dose boost induced antibody with increased somatic hypermutation and higher avidity (19). However, it was unclear whether this effect was due to the delayed boost (0, 1, and 7-month regimen) or the fractional dose. Our study is the first to demonstrate a functional difference in the antibodies induced by the reduced third dose regimen that is associated with protection. However, it is unclear whether the quality of the antibody response is enhanced by a reduced third dose in particular or whether this could be achieved with a reduction of all three doses, which would also have economical and practical advantages. Lower vaccine doses decrease the availability of antigen and therefore could result in greater affinity maturation through increased competition between B cells for T cell help and preferential expansion of B cell clones with the highest affinity B cell receptors (32, 33). Lower doses of antigen at priming also preferentially drive the induction of memory, while higher antigen doses drive differentiation of plasma cells (34). The preferential induction of memory by reducing the priming dose could enhance responses to the subsequent vaccinations and also potentially generate more durable protection. This suggests that the dosing regimen should also be carefully tested to ensure the optimal type of immune response is achieved.

Previous studies have demonstrated that cTfh may be useful biomarkers for GC responses in the absence of lymphoid tissue (35). However, cTfh are a heterogeneous population composed of a number of different subsets, some of which appear to more closely resemble *bona fide* GC Tfh than others (22, 36). The proportions of these subsets have been associated with different diseases: increases in cTfh2/cTfh17 subsets are associated with the production of autoantibodies and disease severity in various autoimmune diseases (22, 37, 38), the development of allergy (39, 40) and the production of broadly neutralizing antibody in HIV^+ individuals (26, 41). By contrast, increases in $CXCR3^+$ cTfh have been implicated in the poor development of humoral immunity against malaria (42, 43) and are proportionally increased in patients with primary immunodeficiencies (44). In addition, a study that observed $CXCR3^+$ cTfh to positively correlate with antibody responses after influenza vaccination also showed that $CXCR3^+$ Tfh that were localized to tonsillar GCs, expressed Fas-L, secreted IFNγ, lacked CD154 expression, and suppressed the activity of GC B cells. Therefore, although this subset was correlated with antibody responses, they were not optimal for their induction (45). In our study, co-administration of RTS,S/AS01B with viral-vectored vaccines led to an increased frequency of $CXCR3^+$ total cTfh compared with RTS,S/AS01B administered alone, and this phenotype was associated with the observed reduction in antibody quantity and quality. Although the observed association was for total cTfh, it would be useful



Percentage of column total	G3/4 CSP Protected	G3/4 CSP Not protected
IL-13	0.33%	0.23%
IFNγ	18.29%	35.84%
IL-6	57.87%	49.89%
IL-10	2.66%	2.20%
IL-2	18.18%	8.58%
TNFα	0.55%	0.53%
IL-22	2.11%	2.73%

FIGURE 5 | Cytokine responses to CSP and MVA. Cytokine responses measured in supernatant after stimulation of 1–2 × 10^6 PBMC at C-1 with CSP or MVA. A multiplex cytokine bead assay (Legendplex, BioLegend) was used to measure a panel of T-helper cytokines. Responses were measured for 19 G1/2 samples stimulated with CSP and 15 G3/4 samples for which there were enough cells to run both CSP and MVA stimulations. **(A)** Heatmap of geomean cytokine concentrations in pg/mL in supernatant of PBMC from G1/2 stimulated with CSP and G3/4 stimulated with CSP or MVA. **(B)** Concentration of IFNγ in supernatant of PBMC stimulated with CSP or MVA, Kruskal–Wallis with Dunn's multiple comparisons $P < 0.0001$. Mann–Whitney analysis between G3 RA-RM-RM and G4 RA-RM-rM $P = 0.0037$. Lower limit of detection for all cytokines in this assay was 1 pg/mL (indicated by the dashed line). **(C)** Proportions of cytokines produced in response to CSP stimulation (geomeans) in G3/4 (R + V) volunteers who were protected compared with those who were not. Table shows the level of each cytokine produced as a percentage of the total cytokine response to CSP in each group. Abbreviations: R G1/2, RTS,S/AS01B vaccinated; R + V G3/4, RTS,S/AS01B and viral-vectored vaccines co-administered; R2V, RTS,S/AS01B and viral-vectored vaccines at a 2-week stagger; A, ChAd63 ME-TRAP; M, MVA ME-TRAP; R, 50 μg third dose of RTS,S/AS01B; r, 10 μg third dose of RTS,S/AS01B; CHMI, controlled human malaria infection; C-1, day before CHMI; CSP, circumsporozoite protein; PBMCs, peripheral blood mononucleocytes; TRAP, thrombospondin-related adhesive protein.

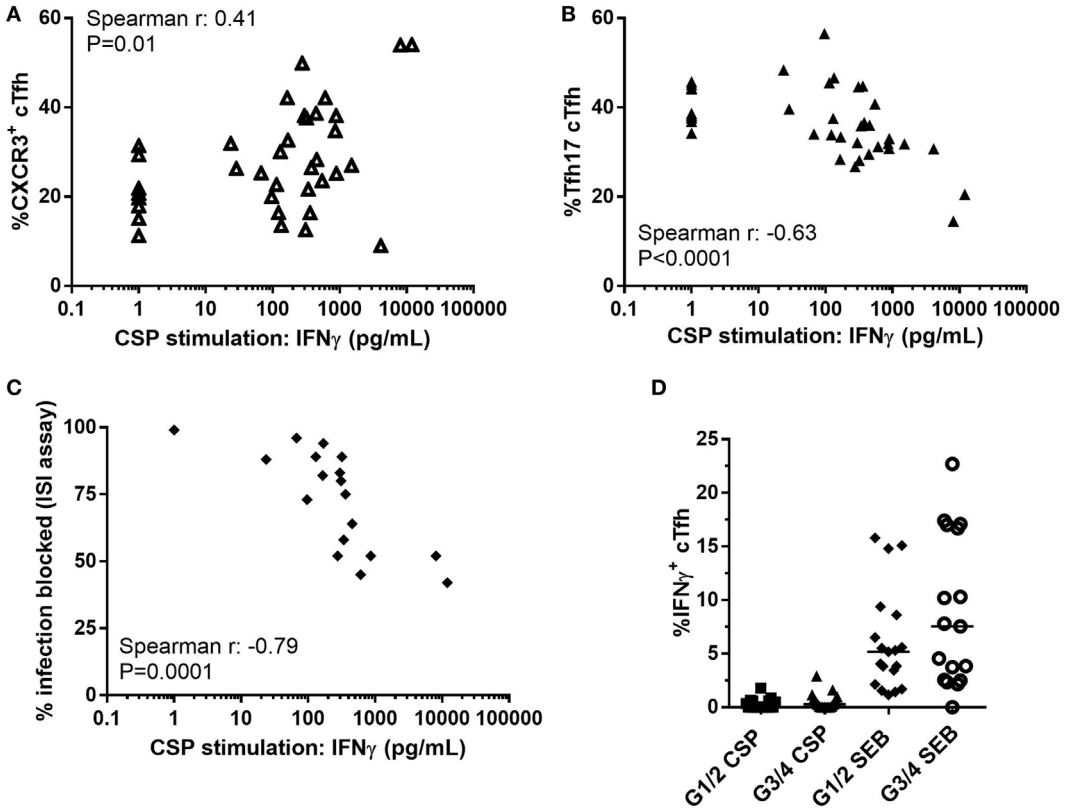


FIGURE 6 | Association between Th1-biased cytokine responses and suppressed humoral immunity. Relationship between concentration of IFNγ in supernatant of PBMCs stimulated with CSP in co-administration trial (G1–4, R, and R + V combined) and: **(A)** Proportion of CXCR3+ cTfh (Spearman *r*: 0.41, *P* = 0.01); **(B)** Proportion of cTfh17 (CXCR3+CCR6−) within cTfh (Spearman *r*: -0.63, *P* < 0.0001); **(C)** Percentage of infection blocked in the ISI assay (Spearman *r*: -0.79, *P* = 0.0001). **(D)** Proportion of IFNγ+ cTfh after stimulation with CSP or SEB, Mann–Whitney analyses between G1/2 (R) and G3/4 (R + V) *P* = 0.14 and *P* = 0.26 for CSP and SEB stimulations, respectively. Abbreviations: R G1/2, RTS,S/AS01B vaccinated; R + V G3/4, RTS,S/AS01B and viral-vectored vaccines co-administered; R2V, RTS,S/AS01B and viral-vectored vaccines at a 2-week stagger; A, ChAd63 ME-TRAP; M, MVA ME-TRAP; R, 50 μg third dose of RTS,S/AS01B; r, 10 μg third dose of RTS,S/AS01B; CHMI, controlled human malaria infection; C-1, day before CHMI; CSP, circumsporozoite protein; cTfh, circulating T follicular helper cell; ISI, inhibition of sporozoite invasion; PBMCs, peripheral blood mononucleocytes; TRAP, thrombospondin-related adhesive protein; SEB, *Staphylococcus* enterotoxin B.

to profile the antigen-specificity of these cells to determine if the increase in CXCR3+ cTfh was due to the induction of cTfh specific for the vector or whether this was a change in the phenotype of the CSP-specific cTfh. There are several methods used to look at antigen-specific cTfh, including cytokine production or CD154 expression after overnight antigen stimulation or the use of antigen-induced markers (45–48). Unfortunately, we found that staining for the chemokine receptors CXCR3 and CCR6 could not reliably be incorporated into these assays (unpublished data). Therefore, we were unable to determine the specificity of the CXCR3+ cTfh.

Circulating Tfh have been shown to produce cytokines, with CXCR3+ cTfh in particular producing IFNγ (49). However, the association we saw between IFNγ in the supernatant and CXCR3+ cTfh was not likely due to production of IFNγ by the CXCR3+ cTfh themselves as only very low percentages of cTfh were observed to produce IFNγ after CSP or SEB stimulation. This suggests that exogenous sources of IFNγ are associated with the polarization of cTfh toward a CXCR3+ phenotype, although vector-specific CXCR3+ cTfh could be a source IFNγ in G3/4 volunteers. MVA has previously been shown to drive a strong IFNγ response, particularly in CD8+ T cells and IFNγ enhances CXCR3 expression on T cells through STAT1 signaling (50–53). It is perhaps therefore unsurprising that MVA induced a CXCR3-skewed cTfh response. However, the impact of this skew on the antibody responses was less predictable, given that CXCR3+ cTfh have in some contexts been positively associated with antibody responses (45, 54) while in other studies they have been associated with suboptimal GC responses and poor humoral immunity (42, 55). In our study when the two different vaccine platforms were co-administered, the IFNγ-dominated cytokine responses driven by viral-vectored vaccines were associated with an increase in CXCR3+ cTfh and a reduction in humoral immunity and protective efficacy. However, if given 2 weeks after RTS,S/AS01B the extent of this CXCR3+ cTfh skew, although only slightly reduced, is no longer associated with a reduction in humoral immunogenicity.

IFNγ induces the production of chemokines CXCL9 (MIG, monokine induced by gamma-interferon), CXCL10 (IP-10, interferon-induced protein of 10 kDa), and CXCL11 (I-TAC,

interferon inducible T cell alpha chemoattractant), which all bind CXCR3. This chemokine system mediates the migration of Th1 $CD4^+$ T cells and cytotoxic T lymphocytes to sites of Th1 inflammation in the periphery (56). MVA has been shown to induce high systemic levels of IP-10 (57). The systemic induction of CXCR3 ligands, in combination with the $CXCR3^+$ cTfh polarization, may result in the reduction of antibody responses by causing an egress of these cTfh from the draining lymph node, preventing them from providing help to B cells in the GC response. A staggered regimen may reduce this effect by allowing time for the RTS,S/AS01B-induced GC response to occur before MVA-induced inflammation begins. Alternative strategies to reduce or avoid the observed immune interference without increasing the number of clinic visits could be to reduce the dose of MVA or to exclude the additional MVA vaccination at week 4, which was included for practical reasons to simplify the vaccination protocol and is not required to induce potent T cell responses (17, 18).

Producing an effective vaccine against malaria will likely depend on a combination of vaccines targeting multiple stages of the parasite lifecycle. In resource-poor settings, mixing, or co-administering, the vaccines will be necessary to reduce the number of clinic visits required, particularly in infants to fit in with the established Expanded Program on Immunisation vaccine schedule. However, the effects of co-administration on immunogenicity and protective efficacy of each vaccine must be carefully examined. Ideally, a combination regimen could be designed to elicit antibody and T cell responses with an additive protective effect and without the immune interference observed here. Examining the cellular mechanisms underlying these differences in antibody responses will be critical for determining how effective, long-lived antibody responses can be induced by vaccination and for informing rational design of vaccine regimens.

ETHICS STATEMENT

All volunteers gave written informed consent prior to participation, and the studies were conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice. The trials were registered with ClinicalTrials.gov (Ref: NCT01883609 and NCT02252640). The study protocols were approved by the UK National Research Ethics Service, Committee South Central—Oxford A (Refs: 13/SC/0208 and 14/SC/0227), the Western Institution Review Board (Ref: 20130698) and the UK Medicines and Healthcare products Regulatory Agency (Refs: 21584/0317/001-0001 and 21584/0333/001-0001). The Local Safety Committee provided safety oversight for both trials and GCP compliance was monitored by the Clinical Trials and Research Governance Team (CTRG) of the University of Oxford.

AUTHOR CONTRIBUTIONS

GB designed laboratory study, interpreted data, and wrote the manuscript. GB and AG acquired and analyzed data. DM, RB, AH, and KE conceived clinical studies and provided project oversight. TR, NV, AH, and KE designed clinical studies. TR and NV conducted clinical work. All the authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01660/full#supplementary-material.

FIGURE S1 | Relationship between NANP-specific isotypes/subclasses and inhibition of sporozoite invasion (ISI). Associations between NANP-specific isotypes and subclass titers at C-1 and ISI **(A)** IgG1, **(B)** IgG2, **(C)** IgG3, **(D)** IgM, and **(E)** IgA.

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Conflict of Interest Statement: AH is a named inventor on patent applications covering malaria vectored vaccines and immunization regimens. DM and RB are employees of GSK, which is developing vaccines for malaria and other diseases. All other authors report no potential conflicts.

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*Pf*s230 and *Pf*s48/45 Fusion Proteins Elicit Strong Transmission-Blocking Antibody Responses Against *Plasmodium falciparum*

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The *Plasmodium falciparum Pf*s230 and *Pf*s48/45 proteins are expressed during transmission from man to mosquito and are leading candidates for a malaria transmission blocking vaccine. Individually they generate transmission blocking (TB) antibodies in rodent models. Whether the single protein vaccines are suitable to use in field settings will primarily depend on their potency to elicit functional antibodies. We hypothesized that a combination of both proteins will be more potent than each protein individually. Therefore we designed chimeric proteins composed of fragments of both *Pf*s230 and *Pf*s48/45 as well as single protein fragments, and expressed these in *Lactoccus lactis*. Both the individual *Pf*s230 and *Pf*s48/45 fragments and chimeras elicited high levels of functional antibodies in mice. Importantly, one of the chimeric proteins elicited over threefold higher transmission blocking antibody responses than the single antigens alone. Furthermore the immunogenicity of one of the chimeras could be enhanced through coupling to a virus-like particle (VLP). Altogether these data support further clinical development of these novel constructs.

Keywords: malaria, vaccines, multivalent, transmission blocking, *Lactococcus lactis*

INTRODUCTION

The transmission of *Plasmodium falciparum* from one person to another relies on the generation of male and female gametocytes in the human host that can be picked up and spread by a mosquito. The aim of a malaria transmission blocking vaccine (MTBV) is to effectively block malaria transmission at the population level thereby contributing to malaria elimination.

Several MTBV candidates have been identified by screening monoclonal antibodies generated against *P. falciparum* mosquito stages for TB activity (1–4). Three proteins, *Pf*s48/45, *Pf*s230, and *Pf*s25 are currently targeted as lead candidates for an MTBV. Of these, *Pf*s48/45 and *Pf*s230 are expressed in the gametocyte as it develops from stage III through V in the human host. Shortly, after being taken up by a blood-feeding mosquito, the parasite emerges from the RBC as a gamete and after a few rounds of replication motile males fertilize female gametes to form zygotes. *Pf*s48/45

is expressed on the surface of both male and female gametes where it is bound to the plasma membrane through a GPI-anchor (5) and forms a stable complex with *Pf*s230 (6). Both *Pf*s48/45 and *Pf*s230 are important for male fertility (7).

Humans develop naturally acquired immunity against *P. falciparum* gametocytes (8–10) and antibodies against *Pf*s230 and *Pf*s48/45 have been associated with TB activity in some but not all immune epidemiological studies (11, 12). Recently, we demonstrated that *Pf*s48/45- and *Pf*s230-specific antibodies exhibit strong TB activity in the standard membrane feeding assay (SMFA) (13), the gold standard for assessing transmission blockade *ex vivo* (2, 14–16). Whether such antibodies act synergistically, as observed for combinations of mAbs targeting different epitopes on *Pf*s48/45 (2), is not yet known.

*Pf*s48/45 and *Pf*s230 are members of the six-cysteine (6-Cys) s48/45 protein family and contain three and fourteen 6-Cys domains respectively (17). Each 6-Cys domain contains up to six cysteine residues that are involved in intra-domain disulfide bond formation which results in conformational antibody epitopes. The C-terminal 6-Cys domain of *Pf*s48/45 contains the conformational epitope I, which is targeted by the most potent TB monoclonal antibody described to date, mAb45.1 (18). We have recently used the *Lactococcus lactis* expression system for the production of the C-terminal 6-Cys domain of *Pf*s48/45 (6C) as a fusion protein (R0.6C) with the N-terminal GLURP-R0 region (19, 20). The resulting fusion protein can be produced in high yields of properly folded monomeric protein which elicited high levels of TB antibodies in small rodents (19, 20). In the case of *Pf*s230, the C fragment spanning the N-terminal pro-domain and first three 6-Cys domains has been shown to elicit the most potent TB antibodies (21). The presence of three 6-Cys domains suggests that disulfide bonds may be critical for proper folding of each of these domains. Accordingly, a series of *Pf*s230-specific transmission-blocking monoclonal antibodies did not recognize reduced *Pf*s230 (22). In an attempt to identify the minimal *Pf*s230-domain involved in the generation of TB antibodies, *Pf*s230 constructs containing the Pro, Pro+I, Pro+I,II, and Pro+I,II,III domains were produced individually in the wheat germ cell-free system (23). Interestingly, the N-terminal Pro domain, which lacks cysteines, was sufficient to elicit complement-dependent TB activity in the SMFA, suggesting that TB antibodies may also be directed against non-conformational epitopes (23). With respect to *Pf*s230, the C-fragment was the first construct to elicit TB antibodies; however, oocyst reduction was incomplete suggesting that folding was compromised by an incorrect cysteine connectivity (21). Another construct, *Pf*s230D1, corresponding to amino acid residues 444 to 736 was produced in *Pichia pastoris* as a properly folded protein and elicited TB antibodies in rodents (24).

While clinical trials with *Pf*s230D1 are ongoing (ClinicalTrials.gov Identifier: NCT02334462) and R0.6C is in early clinical development phase, we sought to identify more potent *Pf*s48/45- and *Pf*s230-based immunogens. We hypothesized that a combination of antibodies against both proteins would be more potent than against each antigen individually. Therefore we constructed chimeric proteins composed of fragments of both *Pf*s230 and *Pf*s48/45, expressed these in *L. lactis* and evaluated antibody responses in rodents. A multicomponent hybrid protein containing both *Pf*s48/45 and *Pf*s230 holds the potential to lower the required threshold of functional antibodies and to reduce the risk of escape mutations.

METHODS

Preparation of Constructs

Three different truncated forms of *Pf*s230 from N-terminus, i.e. Pro (pro domain AA 443 to 590), Pro+I (pro domain and domain I, AA 443 to 736) and Pro+I,II,III (pro domain through domain III, AA 443 to 1132) were amplified by PCR from *P. falciparum* 3D7 DNA (GenBank accession number L08135) and cloned into the *Bgl*II restriction site of pSS5 plasmid containing N-terminus Spycatcher (25). *Pf*s48/45$_{291-428}$ (6C) was amplified from an expression vector encoding R0.6C (19, 20) using the forward primer 5′-CCATGGATCCGAAAAAAAAGTCATACACGGA TGTAACTTC-3′ and the reverse primer 5′-CCATAGATCTT GCTGAATCTATAGTAACTGTCATATAAGC-3′. The amplified PCR product was digested with *Bam*HI and *Bgl*II (underlined) and cloned in frame into plasmids containing the Pro or Pro+I inserts to generate Pro-6C and Pro+I-6C fusion constructs, respectively. All the constructs were verified by DNA sequencing and transformed into *L. lactis* MG1363 by electroporation for expression of recombinant proteins with 6xHis tags.

Fermentation and Protein Purification

Fermentation of *L. lactis* MG1363, containing *Pf*s230 or *Pf*s230-*Pf*s48/45 fusion constructs were carried out as described previously (19, 26). Briefly, cell-free culture-filtrates were concentrated five-fold and buffer exchanged into Tris buffer (50 mM Tris, 50mM NaCl pH 8.0 supplemented with 10mM Imidazole) using a Quix Stand Benchtop system (Hollow fiber cartridge with cutoff at 10,000 or 30,000 Da, surface area 650cm^2, GE Healthcare, Sweden) followed by filtration through a Durapore filter (PVDF, 0.22 μm, Millipore) and applied to a 5 ml HisTrap HP column (GE Healthcare, Sweden). Bound protein was eluted with 500mM Imidazole in Tris buffer pH 8.0 (50 mM Tris, 50mM NaCl) at a flow rate of 4 ml/min. Fractions containing the desired protein were further applied to a 5 ml HiTrap Q HP column (GE Healthcare, Sweden) for purification of monomeric proteins. Bound protein was eluted through step gradient elution in Tris buffer pH 8.0 (50 mM Tris, 1mM EDTA, 1 M NaCl) and fractions containing monomers were concentrated by a VIVA spin column with a 10 or 30 kDa cutoff (Vivascience, Germany), and kept in 50 mM Tris, 250 mM NaCl and 1 mM EDTA, pH 8.0 at −80°C until use. Immune purification for Pro-6C and Pro+I-6C was done as previously described (26). Fractions containing the desired protein were pooled and then concentrated and buffer exchanged against 50 mM Tris, 100 mM NaCl, and 1 mM EDTA, pH 8.0 and kept at −80°C until use. Fractions were analyzed by SDS-PAGE and immune blotting with mAb45.1 against *Pf*s48/45 conformational epitope I. Protein concentrations were measured using a BCA kit (Thermo Fisher Scientific, USA).

Protein Characterization

Analytical size exclusion high-performance liquid chromatography (SE-HPLC) of purified fusion proteins was performed as described previously (19, 20). Briefly, 5 µl of protein was loaded on an Agilent advance Bio SEC 300 Å, 2.7 µm, 4.6 × 300 mm SEC column (Agilent Technologies, GB) and eluted with a 0.1 ml/min flow of elution buffer (phosphate buffer) at room temperature. Protein standards (Sigma Aldrich) were also run using the same conditions mentioned above for sizing of the purified recombinant proteins. The amount of free cysteine residues was measured using Ellman's Reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions. A standard curve was constructed using known concentrations of free cysteine (Sigma-Aldrich, USA). Folding was determined in the mAb45.1 sandwich ELISA as described (19, 26).

Production of VLPs

SpyTag was genetically fused to the N-terminus of AP205, as previously described (27). In brief, the SpyTag peptide sequence (AHIVMVDAYKPTK) was fused to the gene sequence encoding the major AP205 coat protein (Gene ID: 956335) using a flexible linker (GSGTAGGGSGS) between the two sequences. The SpyTag-AP205 VLPs were expressed in *Escherichia coli* One Shot® BL21 Star™ (DE3) cells (Thermo Fisher Scientific, USA) and purified by ultracentrifugation using an Optiprep™ (Sigma-Aldrich, USA) gradient. For conjugation to VLPs, purified soluble Pro-6C or Pro+I-6C proteins were incubated at a molar ratio of 1:1 (VLP/antigen) in a 1xPBS buffer for 2 h at room temperature. Unbound protein was removed by dialysis against PBS using 1,000 MWCO dialysis tubing (Spectrum Labs, USA). Densitometric analysis of SDS-PAGE gels was used to estimate protein concentrations.

Dynamic Light Scattering

Uncoupled VLP, soluble proteins and proteins conjugated to VLP were adjusted to 0.5–1 mg/ml in PBS and spun at 15,000 g for 10 min. Seventy Microliter sample was loaded into a disposable Eppendorf Uvette cuvette (Sigma-Aldrich, USA) and measured at 25°C on a DynoPro NanoStar (WYATT Technology, USA) equipped with a 658 nm laser. Each sample was measured 20 times and intensity-average size and percentage polydispersity (PD) was estimated using Dynamic software (Version 7.5.0).

Electron Microscopy

Pro-6C or Pro+I-6C coupled to VLP (with concentrations between 0.4 and 0.5 mg/ml based on antigen content) were incubated on carbon-coated and glow-discharged grids and negatively stained with 2% phosphotungstic acid (pH 7.4). The particles were analyzed on a CM 100 BioTWIN electron microscope with an accelerating voltage of 80 kV. Images were acquired using an Olympus Veleta camera and particle size was estimated using iTEM software.

Animals and Immunogenicity Studies

In the first experiment, groups ($n = 5$) of CD-1 mice 5–7 weeks of age (Janvier Labs, Denmark) were immunized 3 times at 3-week interval by the intramuscular injection of equimolar amounts of immune-purified Pro-6C and the individual *Pf*s230 and *P*fs48/45 recombinant protein constructs formulated with Alhydrogel® (Brenntag, Denmark) to a final concentration of 2 mg/ml Aluminum. Please note that six mice received R0.6C. Each dose contained 128 pmoles of soluble protein (equivalent to 2 µg 6C). Serum was collected on days 14, 35, and 56. In the second experiment, groups ($n = 8$) of CD-1 mice were immunized with 64 pmoles (equivalent to 1 µg 6C) Pro-6C or Pro+I-6C (soluble or conjugated to VLP) as described above for the first experiment. One mouse receiving Pro+I-6C was terminated due to behavioral abnormalities not related to vaccination. All animals were treated in accordance with the regulations and guidelines of the European and National authorities.

Enzyme-Linked Immunosorbent Assay (ELISA) for Antibody Response Measurement

Gametocyte extract ELISA was performed with cultured sexual stage of *Pf* NF54 parasites as previously described (26). Serum immunoglobulin (IgG) subclass levels were measured using ELISA as previously described (28). For antigen-specific ELISA, 96-well plates (Nunc MaxiSorp) were coated with 0.5 µg/well of *Pf*s48/45-6C (25), Pro+I, or Pro+I,II,II as appropriate. Antigen-specific antibodies were detected using HRP-conjugated polyclonal goat anti-mouse IgG (Novex A16072, diluted 1:3000). Antibody midpoint titer (EC50) was calculated using sigmoidal curve fitting. One-sided analysis of variance on the log-transformed values was used to confirm that ELISA data contain essential differences. Mann-Whitney test was then used to investigate whether the chimera elicited higher levels of specific antibodies compared to individual components. *p*-Values are two-sided and quoted without adjustment for multiple testing since the significance of the chimera was the primary problem under investigation. *p*-values ≤ 0.05 were considered significant. Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software, USA).

Standard Membrane Feeding Assay (SMFA)

The biological activity of specific antisera was assessed in the SMFA as previously described (26, 29). Depending on availability, wild type *P. falciparum* NF54 gametocytes or transgenic *P. falciparum* NF54 (NF54-HGL) gametocytes expressing luciferase (29) were fed to *Anopheles stephensi* mosquitoes that were reared and maintained at Radboudumc, The Netherlands. Non-heat inactivated mice sera, and active or heat-inactivated human complement was added to the cultured material prior to feeding to mosquitoes. After 6–8 days, oocysts in 20 fed mosquitoes were counted by microscopy, or quantified in four pools of five mosquitoes (NF54-HGL) by measuring luciferase levels (29). Samples were tested in two independent SMFA experiments. Luminescence-based TRA estimates from two independent feeds with each of four pools of five mosquitoes were made using generalized linear mixed models (GLMMs) without zero-inflated negative binomial error structure (30, 31). Microscopy-based estimates from two

feeds with 20 mosquitoes each were made using GLMMs with zero-inflated negative binomial error structure (30, 31). Statistical differences between test samples were determined using General Linear Mixed Regression analysis. Statistical analyses were performed using R studio (v. 3.2.4, The R Foundation, Boston, USA). Pre-immune pooled mice serum samples were also tested in a single independent SMFA experiment (**Figure S2**).

Depletion of Antigen-Specific IgGs

*Pf*s230- and *Pf*s48/45-specific antibodies were depleted from serum using *Pf*s230-CMB (32) and R0.10C-containing columns respectively, as previously described (13). The *Pf*s230-CMB fragment contains AA 444-730 and thus covers the Pro+I fragment expressed in *L. lactis*. To test if all antigen-specific IgGs were depleted, the flow through serum (depleted serum) was tested in ELISA using plates coated with *Pf*s230-CMB or SpyC-6C as appropriate (25, 26).

RESULTS

Expression of a Multivalent *Pf*s230-*Pf*s48/45 Chimera in *L. Lactis*

To test whether a multivalent vaccine targeting *Pf*s48/45 and *Pf*s230 is immunogenic, we generated a chimeric construct containing the Pro domain of *Pf*s230 fused to the 6C fragment of *Pf*s48/45 (**Figure 1A**). We anticipated that this Pro-6C fusion protein would express well in *L. lactis* since the *Pf*s230-Pro domain is glutamate-rich, does not contain cysteines, and is similar to the R0 domain which previously enhanced expression of properly-folded 6C in *L. lactis* (25, 26). In addition to Pro-6C, we made constructs that either contained *Pf*s48/45 or *Pf*s230 fragments (**Figure 1A**). *L. lactis* MG1363 harboring these constructs were grown in a 1L bioreactor and the respective recombinant proteins were purified from the clarified supernatant through the C-terminal His-tag by immobilized metal affinity chromatography and ion exchange chromatography (**Figure 1B**). As expected, protein yields decreased with increasing number of *Pf*s230 domains

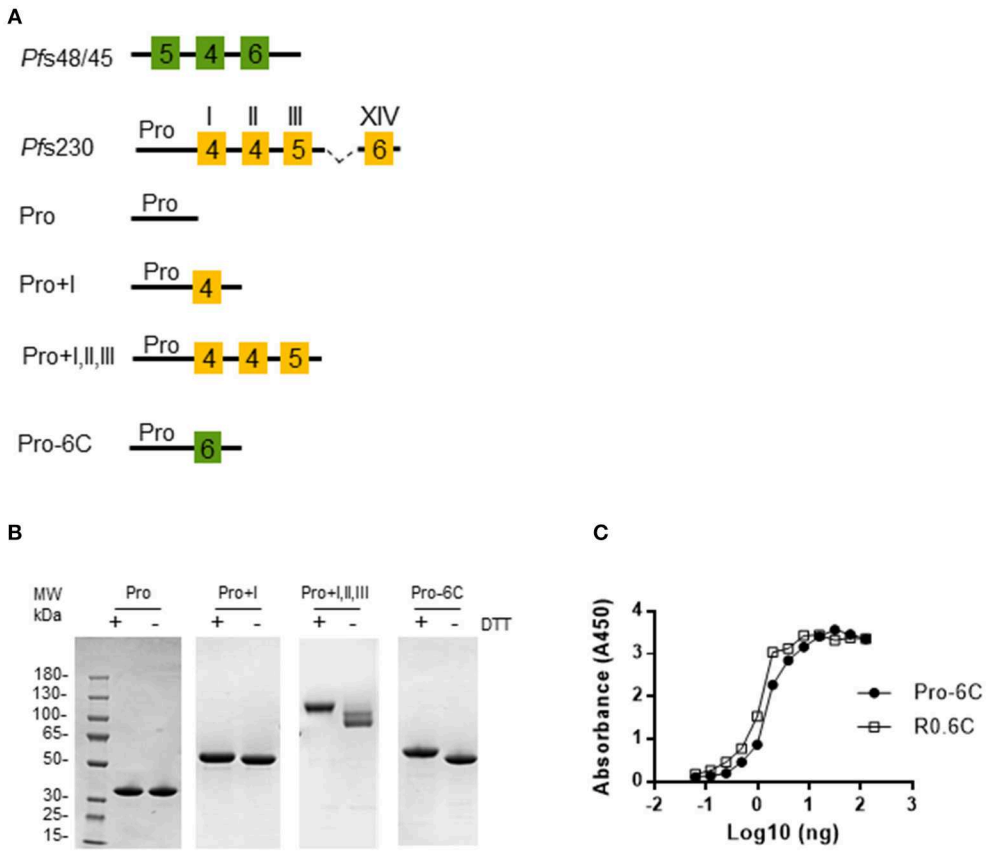

FIGURE 1 | Production of recombinant *Pf*s230 and *Pf*s48/45. **(A)** Schematic representation of *Pf*s230 constructs and *Pf*s230-*Pf*s48/45 chimera. Each construct contains the SpyCatcher sequence at the N-terminus and a His-tag at the C-terminus. 6-Cys domains are shown as boxes and numbers indicate the number of cysteines. The Pro-domain does not contain cysteines. **(B)** Coomassie blue stained 4–12.5% polyacrylamide gel of conventionally purified *Pf*s230 constructs and immune-purified Pro-6C chimera. Protein was loaded in each lane with (+) or without (–) DTT (10 mM). The sizes (kDa) of the molecular mass markers are indicated. **(C)** Sandwich ELISA of purified Pro-6C chimera. The antigens were captured with mAb45.1 and detected with anti-His-HRP. Immune purified R0.6C were used as a reference. X-axis is shown on a logarithmic scale.

(**Table S1**). Pro-6C was further immune-purified on a mAb 45.1-column to enrich for properly folded protein species (**Figure 1B**). The yield of immune-purified Pro-6C was 15 mg/L, similar to that of R0.6C. Conformational mAb 45.1 against the *Pf*s48/45 epitope I reacted with Pro-6C and this binding was equivalent to that of immune-purified R0.6C, suggesting that they exhibit similar cysteine-connectivity (**Figure 1C**).

Immunogenicity of Soluble *Pf*s48/45 and *Pf*s230 Protein Constructs

Groups of mice were immunized 3 times at 3-week interval with equimolar amounts of Pro-6C and individual *Pf*s230 and *Pf*s48/45 recombinant protein constructs formulated on Alhydrogel®. We used a suboptimal antigen dose to detect differences in immunogenic properties between protein constructs. Chimeric Pro-6C elicited significantly higher levels of gametocyte-specific antibodies than those obtained with the individual Pro domain (Mann-Whitney test, $p = 0.0079$) and levels comparable to those obtained with Pro+I, and R0.6C (**Figure 2A**). We found that levels of specific antibodies against the Pro and 6C domains were similar in mice immunized with Pro-6C compared to mice immunized with the individual Pro and 6C (R0.6C) antigens, suggesting that these domains do not exhibit antigenic competition (**Figures 2B,C**). As expected, levels of *Pf*s230-specific antibodies increased with *Pf*s230 fragment length (**Figure 2C**). The functional activity of vaccine-induced antibodies was determined by testing pooled antisera from each group in serial dilutions in the SMFA. All proteins except the Pro domain, elicited a TB response of >80% at a 1/9 dilution. Interestingly, Pro-6C induced higher levels of functional

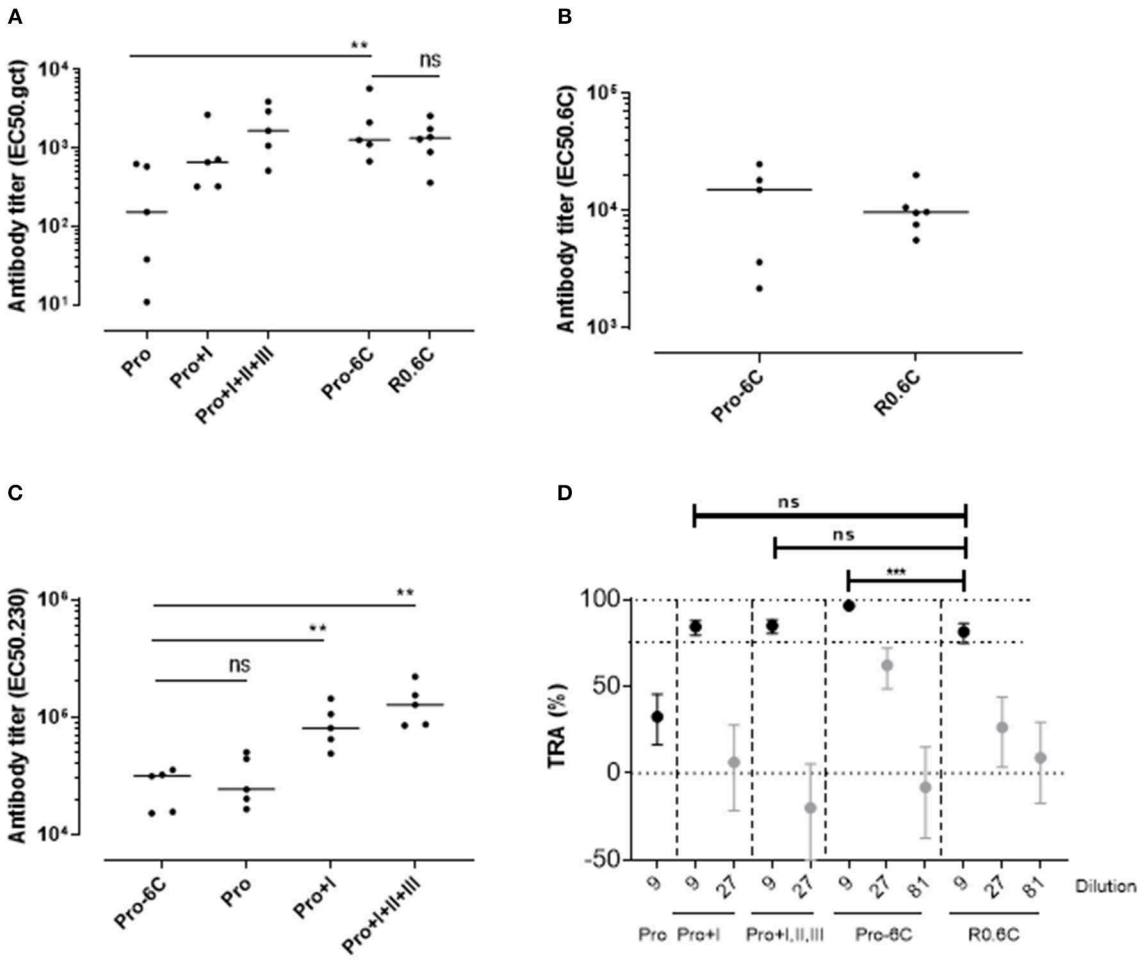

FIGURE 2 | Immunogenicity of Pro-6C and individual fragments. Groups of mice ($n = 5$) were immunized with immune purified Pro-6C in a comparative study with individual Pro, Pro+I, Pro+I,II,III and R0.6C constructs. Day 56 serum was tested for antibody reactivity on ELISA plates coated with **(A)** gametocyte extract, **(B)** *Pf*s48/45-6C or **(C)** *Pf*s230 Pro+I,II,III. Antibody titers (dots represent individual mice) are expressed as EC50 values. Horizontal lines represent median values. The asterisks represent statistical significance determined by Mann-Whitney test (**$p < 0.01$, ns not significant). **(D)** Functional activity of serial dilutions of pooled sera in the SMFA. Transmission reducing activity (TRA) is the reduction of oocyst numbers compared to a serum control. All samples were tested in the presence of active complement. Data points are best estimates of two independent SMFA experiments and error bars represent 95% confidence intervals. Comparison between test samples was done using General Linear Mixed Regression analysis (***$p < 0.005$, ns, not significant).

antibodies than the other recombinant proteins including R0.6C ($p < 0.001$) (**Figure 2D**), supporting further investigation of multi-component *Pf*s230-*Pf*s48/45 vaccines.

Generation of Soluble and VLP-Based Chimeric Constructs

Next, we tested whether the potency of Pro-6C could be further increased by including the first 6-Cys domain of *Pf*s230 (Pro+I-6C) (**Figure 3A**). The Pro+I-6C chimera was purified following the same workflow developed for Pro-6C. The yield of immune-purified Pro+I-6C was 5 mg/L, which was 3-fold lower than that of Pro-6C most likely due to the additional cysteine residues (**Table S1**). The folding of both chimera was similar as determined in the mAb45.1 sandwich ELISA (**Figure 3B**). Disulfide-bonding was confirmed by demonstrating very low levels of free thiol groups (<1%) under native conditions (data not shown). Immune purified Pro-6C and Pro+I-6C eluted as single peaks by analytical size exclusion chromatography demonstrating that they form homogeneous solutions of monomeric protein species (**Figures 3C,D**). Before starting *in vivo* immunogenicity studies with both soluble constructs, we also coupled Pro-6C and Pro+I-6C to virus-like particles (VLPs) to see if this would further increase the immunogenicity of the chimeras. Both Pro-6C and Pro+I-6C contained a SpyCatcher domain allowing covalent coupling to SpyTag-decorated AP205 VLPs (25, 33). Spy-Catcher Pro-6C and Pro+I-6C coupled to SpyTag VLPs efficiently (**Figure 4A**) and properly folded *Pf*s48/45 epitope I was retained during conjugation, as shown by western blot and mAb45.1 sandwich ELISA (**Figures 4A,B**). Both VLPs formed homogenous populations of non-aggregated antigen-VLP complexes as demonstrated by transmission electron microscopy (**Figure 4C**). Furthermore, the VLP-particles displaying Pro-6C and Pro+I-6C demonstrated a low percentage of polydispersity (<16%) measured by dynamic light scattering (DLS) experiments and an average size of 71.8 and 73.7 nm, respectively (**Figure 4D**).

Immunogenicity of Soluble and VLP-Based Chimeric Constructs

The immunogenicity of soluble Pro-6C and Pro+I-6C, and of Pro-6C and Pro+I-6C coupled to VLPs was assessed *in vivo*.

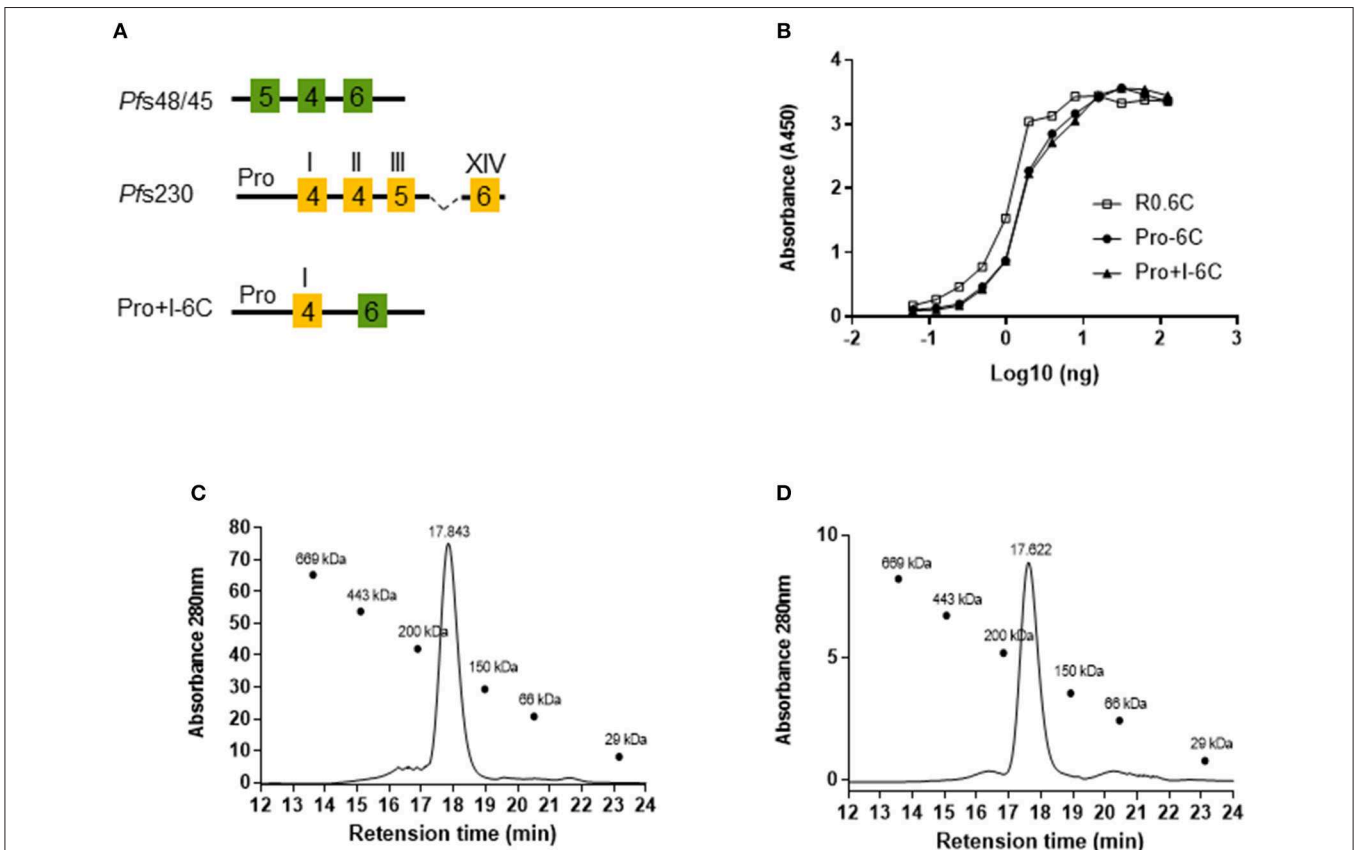

FIGURE 3 | Design and characterization of a multi-domain *Pf*s230-*Pf*s48/45 chimera. **(A)** Schematic representation of the Pro+I-6C chimera. This protein contains the SpyCatcher sequence at the N-terminus and a His-tag at the C-terminus. 6-Cys domains are shown as boxes and numbers indicate the number of cysteines. The Pro-domain does not contain cysteines. **(B)** Sandwich ELISA of purified chimera. Immune purified R0.6C was used as a reference. Size exclusion chromatography analysis of **(C)** Pro-6C and **(D)** Pro+I-6C. SE-HPLC was performed under native conditions in a phosphate buffer of pH 7.2 to determine the amount of monomer in the sample. The sizes (kDa) of the molecular mass markers are indicated.

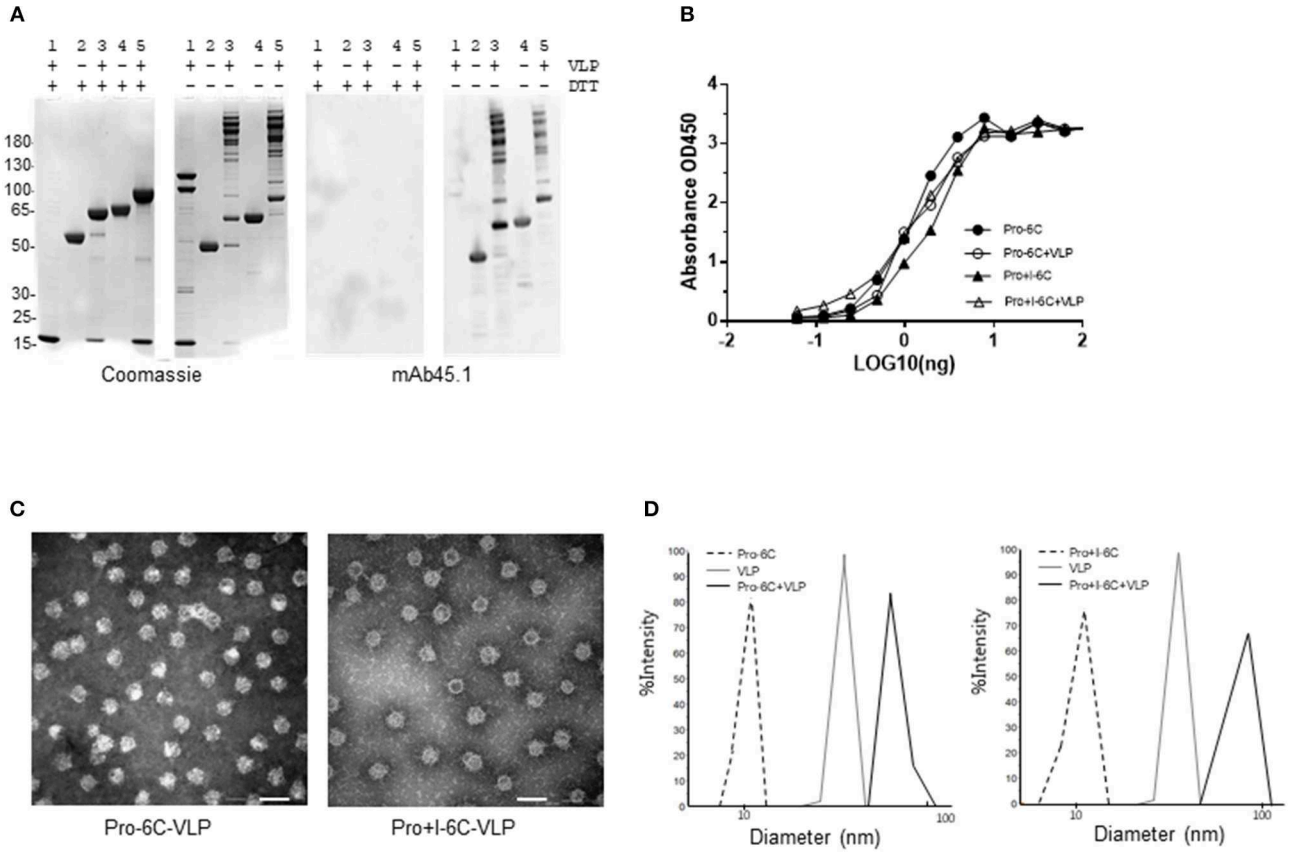

FIGURE 4 | Characterization of virus-like particle-based vaccines. SpyCatcher tagged Pro-6C and Pro+I-6C were mixed with SpyTag-AP205 resulting in a unidirectional display of the chimeras on the VLP surface. **(A)** Reduced and non-reduced SDS-PAGE gel and western blot. The gels (left) are stained with coomassie blue, while the western blots (right) are developed with mAb45.1 as primary antibody. The following was loaded corresponding to the numbers; Lane 1: AP205 (VLP); lane 2: Pro-6C; lane 3: Pro-6C-VLP; lane 4: Pro+I-6C; Lane 5: Pro+I-6C-VLP. **(B)** Sandwich ELISA, using mAb 45.1 as the solid phase capture antibody. **(C)** Transmission electron microscopy images (negative stain) of the VLP-based vaccines after assembly. Both Pro-6C-VLPs and Pro+I-6C-VLPs appear non-aggregated, uniformly dispersed and have an estimated size of 30 nm. Scale bar 100 nm. **(D)** Dynamic light scattering (DLS) profile of the vaccine components Pro-6C [10.5 nm, polydispersity (PD) 10.7%], Pro+I-6C (10.8 nm, PD 20.7%), VLP (25.6 nm, PD 16.8%) and the purified vaccine products; Pro-6C-VLP (71.8 nm, PD 11.5%), and Pro+I-6C-VLP (73.7 nm, PD 15.8%).

Groups of CD-1 mice ($n = 8$) were immunized 3 times at 3-week intervals with equimolar amounts of antigen adjuvanted on Alhydrogel®. Soluble Pro+I-6C elicited significantly (Mann-Whitney test, $p = 0.0079$) higher levels of *Pf*s230-specific responses than soluble Pro-6C (**Figure 5A**). However, this increase was not associated with higher levels of gametocyte-specific antibodies (**Figure 5C**), possibly due to the difference in Pfs230-specific antibody levels being masked by higher 6C-specific signals in the gametocyte ELISA, as observed for single antigen constructs (**Figure 2A**). The functional activity of pooled anti-sera from each group was then tested at serial dilutions in the SMFA. Antibodies against soluble Pro+I-6C promoted higher SMFA activity than antibodies against Pro-6C at a 1/27 dilution ($p < 0.001$) (**Figure 5D**), in line with the SMFA results obtained with the single domain constructs (**Figure 2D**). VLP display did not provide an increase in gametocyte-, *Pf*s48/45-, or *Pf*s230-specific antibodies for both Pro-6C and Pro+I-6C (**Figures 5A–C**). VLP-display of Pro-6C enhanced (Mann-Whitney test, $p < 0.001$) the production of functional antibodies as demonstrated in the SMFA while there was no such effect for Pro+I-6C (**Figure 5D**).

Functional Activity of Domain Specific Antibodies in the SMFA

To investigate the functional activity of domain-specific antibodies, pooled sera from mice immunized with soluble Pro+I-6C (no VLP) were depleted for *Pf*s230- and *Pf*s48/45-specific antibodies using affinity columns with immobilized *Pf*s230 and *Pf*s48/45 respectively, as previously described for human antibodies (13). Antibody depletion was confirmed by ELISA (data not shown). The functional activity of antibody-depleted sera was then tested in the SMFA in 2-fold serial dilutions in the presence of complement. Sera depleted of *Pf*s230-specific or *Pf*s48/45-specific antibodies retained transmission blocking activity at 9- and 18-fold dilutions, respectively, demonstrating that functional antibodies against both proteins are induced by the Pro+I-6C construct

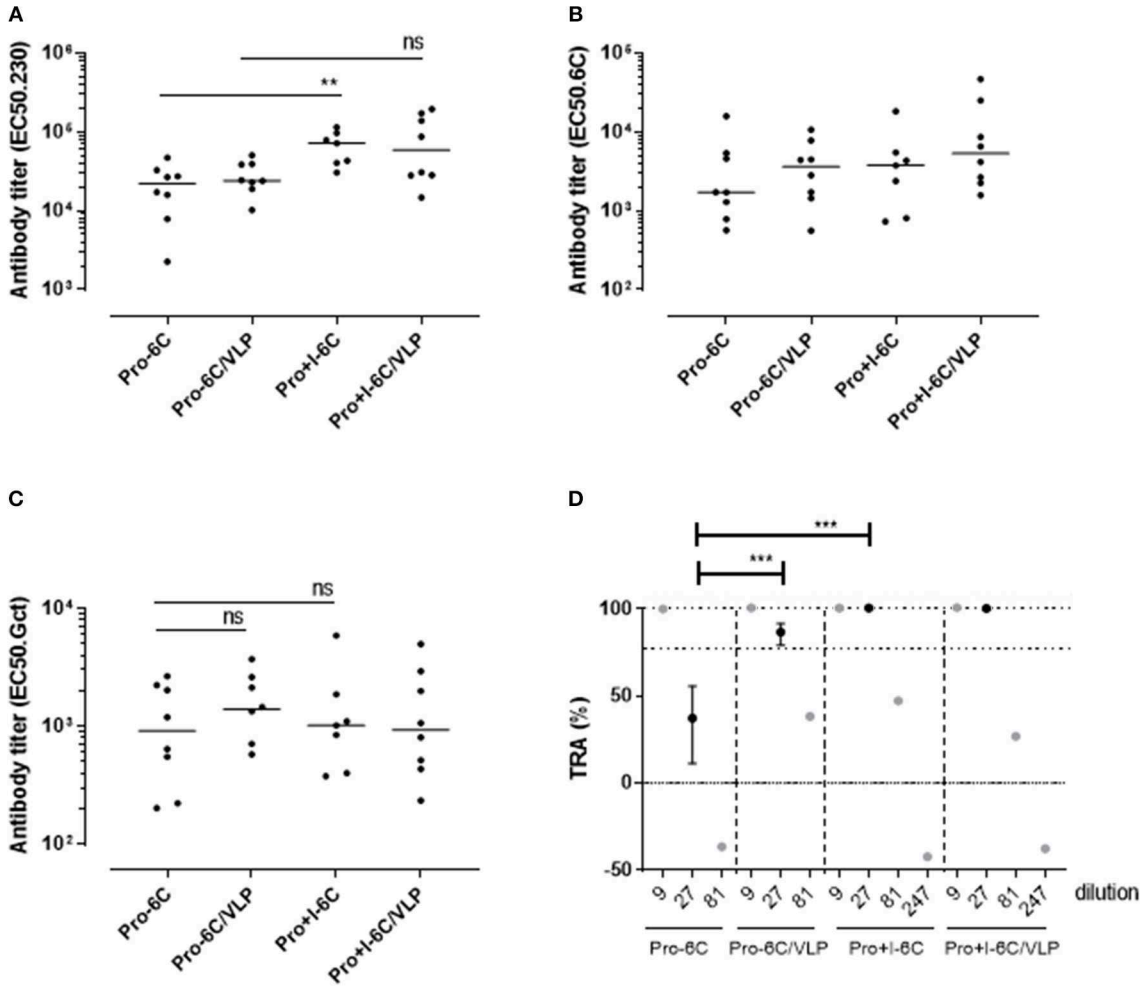

FIGURE 5 | VLP-delivery of Pro-6C and Pro+I-6C. Groups of mice ($n = 8$) were immunized with soluble Pro-6C and Pro+I-6C or bound to AP205. Day 56 serum was tested for antibody reactivity on ELISA plates coated with **(A)** *Pf*s230 Pro+I,II,III **(B)**, *Pf*s48/45-6C or **(C)** gametocyte extract. Antibody titers (dots represents individual mice) are expressed as EC_{50} values. Horizontal lines represent median values. The asterisks represent statistical significance determined by Mann-Whitney test (**$p < 0.01$, ns not significant). **(D)** Functional activities of serial diluted sera were assessed in the SMFA. Transmission reducing activity (TRA) is the reduction of oocyst load compared to a serum control. All samples are tested in the presence of active complement. Data points are best estimates of two independent SMFA experiments and error bars represent 95% confidence intervals. Note that 1/81 and 1/247 samples were tested in SMFA only once and therefore no confidence intervals are given. Comparison between samples was done using General Linear Mixed Regression analysis (***$p < 0.005$, ns, not significant).

(**Figure 6A**). Since the TB activity of *Pf*s230-specific antibodies depends on complement (22), depleted anti-sera were re-tested in SMFA with and without active complement. As expected, *Pf*s230-specific antibodies (Δ6C) lost their functional activity in the absence of active complement (**Figure 6B**). Since complement fixation is dependent on specific antibody subclasses (34), we determined the IgG subclass profile elicited by the Pro+I-6C formulation. The chimera elicited predominantly IgG1 antibodies, and to a lower extent IgG2a and IgG2b antibodies (**Figure S1**). Altogether, these data show that functional antibodies are generated against both domains of the chimeric construct and that *Pf*s230-specific antibodies are complement dependent.

DISCUSSION

*Pf*s230 and *Pf*s48/45 are expressed during the *P. falciparum* sexual stages in humans and elicit antibodies which effectively prevent parasite multiplication in the infected mosquito (13). Promising *Pf*s230- and *Pf*s48/45-based MTBV candidates are currently entering clinical development individually. Here we set out to produce constructs with higher potency and to this end produced and tested chimeric proteins based on both vaccine candidates. To the best of our knowledge, this is the first study that explores a multivalent MTBV based on *Pf*s230 and *Pf*s48/45. Our chimeric proteins induce antibodies that have >80% transmission reducing activity in the SMFA, which is high

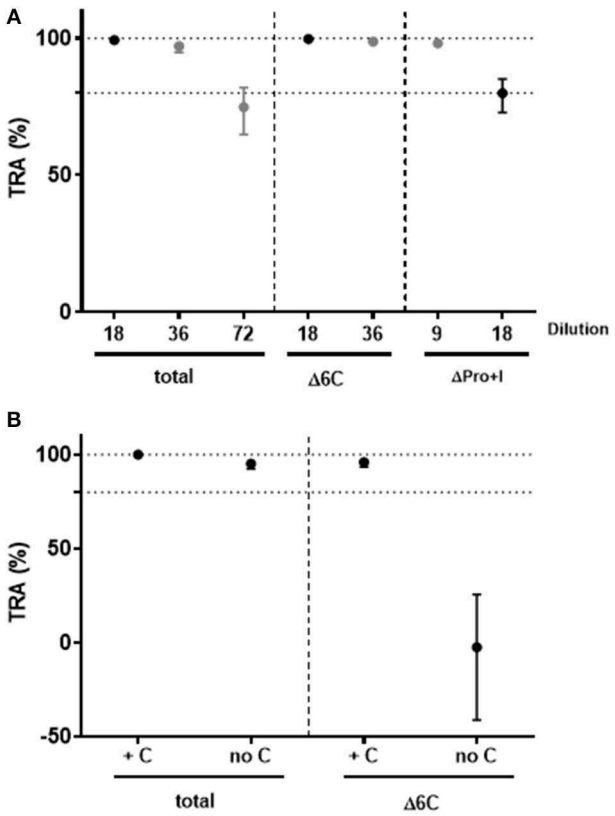

FIGURE 6 | Functional activity of domain-specific antibodies generated by Pro+I-6C. Domain-specific antibodies were depleted from pooled sera of mice immunized with Pro+I-6C, using columns containing *Pfs*230-Pro+I and *Pfs*48/45-10C (13), to generate sera that recognize either 6C or Pro+I respectively. **(A)** Serial dilutions of sera containing Pro+I antibodies only (Δ6C) and sera containing 6C antibodies only (ΔPro+I) were tested in the SMFA, in the presence of active complement. **(B)** Total sera and sera containing Pro+I antibodies only were re-tested with (+C) and without (no C) active complement at a dilution of 1/9. Transmission reducing activity (TRA) is the reduction of oocyst numbers compared to a serum control. Data points are best estimates of two independent SMFA experiments and error bars represent 95% confidence intervals.

enough to meet the no/go decision criterion for selection and further vaccine development (35). Overall, our data show that these chimeric proteins elicited antibodies with higher TB activity in the SMFA than the single proteins alone (**Figures 2D**, **5D**) and are therefore attractive next generation vaccine candidates.

One concern when generating multivalent vaccines is that one of the components is immunodominant and that responses against the other component are therefore compromised (36, 37). To investigate this we tested specific antibody responses against the individual domains by ELISA and demonstrated that these are not affected when the domains are presented as part of chimeric proteins. Moreover, the depletion experiments showed that antibodies against the Pro+I- and 6C-domains are functional in the SMFA. Interestingly, the Pro+I-6C chimera elicited higher levels of functional *Pf*s230-specific antibodies than the Pro+I domain alone; sera from Pro+I immunized mice showed no TRA in the SMFA at 1/27 dilution, whereas sera depleted of 6C-specific antibodies from mice immunized with Pro+I-6C still retained 99% TRA at 1/36 dilution. This apparent difference was not reflected in levels of specific antibodies detected in the *Pf*s230-ELISA indicating that functional activity in the SMFA is not only dependent on quantity but also the quality of antibodies. Importantly, there was no difference in levels of functional antibodies against the *Pf*s48/45-6C-domain when comparing Pro+I-6C and R0.6C immunized mice suggesting that the increase in functional activity of *Pf*s230-specific antibodies is, at least in part, related to a better presentation of antibody epitopes in the *Pf*s230 domain I of the Pro+I-6C chimera.

Adjuvants with the ability to enhance antigen immunogenicity are critical components of an efficacious subunit vaccine. In the case of chimeras that include *Pf*s230, it is particularly important that vaccination elicit antibodies with complement-fixing activity. Here we show that Pro+I-6C formulated on Alhydrogel® elicited high levels of IgG1 antibodies, but also Pro+I specific IgG2a and IgG2b antibodies in mice. Murine IgG2a and IgG2b are subclasses of IgG that bind with high affinity to human complement (34). The activation of complement may subsequently trigger lysis of gametes in the infected mosquito (22). It remains to be determined whether the Pro+I-6C chimera may also elicit complement-fixing antibodies in humans.

Both *Pf*s230- and *Pf*s48/45-based vaccine constructs are rich in cysteine and proper disulfide bond formation is critical for functional antibody responses (22, 38). Therefore, successful construction and production of chimeric proteins depend on the maintenance of conformational integrity of immunologically relevant regions of the individual domains. The mAb45.1 is a conformational mAb (2) that reacts with properly folded *Pf*s48/45 but not disulphide reduced *Pfs*48/45 (38). Its reactivity with the fusion proteins indicate proper cysteine connectivity of the *Pf*s48/45 domain. Accordingly, our results from ELISA and immunoblotting analysis showed that the purified chimeras expressed in *L. lactis* were strongly recognized by mAb45.1 and had thus retained conformational epitope I in the *Pf*s48/45-6C domain. It was particularly encouraging that recombinant Pro+I-6C reacted with mAb45.1 since *Pf*s230 domain I contains four cysteine residues which may potentially interfere with disulfide bonding of the *Pf*s48/45-6C domain. While disulphide bond studies have not been completed on the two chimera, the positive reactivity with mAb45.1, indicate that the *Pf*s230 sequence does not disrupt the proper disulphide formation of the *Pf*s48/45 6C domain contained within. Correct folding was further supported by antibody depletion experiments showing that both domains of the chimeras elicited functional antibodies. The data thus demonstrate that *L. lactis* is not only suitable for expression of *Pf*s48/45 fragments (19), but also for *Pf*s230 fragments and fusions thereof. The immune purification of the two chimeras used here is not compliant with cGMP manufacturing since a monoclonal antibody of rat origin was used. However, a purification process based on conventional chromatographic procedures is currently being developed. Although yields and purity remain to be determined, it is likely that high product

yields can be obtained through upscaling the fermentation process which is straightforward since there is no requirement for oxygen and vigorous stirring during fermentation (39). Additionally, yields of properly folded protein species may also be increased through protein refolding processes as those developed for R0.6C (20).

In conclusion, we have produced two chimeras composed of leading vaccine candidates against the transmission stages of *P. falciparum*. Both chimera elicited high levels of functional antibodies in rodents and outperformed the corresponding individual protein fragments. Previously, rodents have been immunized with *Pf*s25 administered together with either *Pf*s28 or *Pf*s230C (40). In contrast to our findings for Pfs48/45-Pfs230 chimeras, the *Pf*s25-based dual-antigen vaccines did not elicit higher levels of functional antibodies than the corresponding single antigen vaccines (40). Together these data demonstrate that additive effects can only be achieved for certain antigen combinations. Our results do not only support the use of chimeric proteins for MTBV development but also for malaria vaccine development in general (26, 28, 41, 42). Another advantage of multi-component vaccines is that the production of antibodies against multiple antigens might help reduce the spread of potential escape mutants in the population. Such escape mutants may compromise overall vaccine efficacy as exemplified with the RTS,S malaria vaccine (43). This multivalent strategy is thus conceptually attractive and will constitute a novel means toward control and eventually eradication of malaria once clinical efficacy has been demonstrated.

ETHICS STATEMENT

The animal studies were approved by the Danish Animal Experiments Inspectorate. Approval number: 2013-15-2934-00902/BES.

AUTHOR CONTRIBUTIONS

SS, ST, BC, KT, RS, WG, G-JvG, MvdV-B, and MJ: performed the experiments. ST, MN, AS, and AFS: designed the VLP constructs. SS, MT, and MJ: designed the experiments. MT, MJ, and RWS: wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01256/full#supplementary-material

Figure S1 | Subclass-specific antibody responses in mice immunized with Pro+I-6C. Day 56 sera were tested for antibody reactivity on ELISA plates coated **(A)** Pro+I or **(B)** 6C. Results for individual mice are shown, and a horizontal bar represents each median value.

Figure S2 | Pre-immune mouse serum contains low intrinsic TRA. Pooled mouse serum from each group was tested at 1/9 dilution in a single SMFA experiment. TRA is the reduction of oocyst numbers compared to a serum control. Error bars represent 95% confidence intervals. TRA values below 50% have large confidence intervals and this can explain the observed variation between different groups. The mean TRA of all groups is 21% (CIs: 5.7–34.1%) demonstrating that mouse serum has low intrinsic TRA.

Table S1 | Production and quantification of *Pf*s230 and *Pf*s230/*Pf*s48/45 chimeric protein in *L. lactis*.

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Conflict of Interest Statement: A patent application covering the VLP spy-technology has been filed by the University of Copenhagen.

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REVIEW
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Immune Responses to the Sexual Stages of *Plasmodium falciparum* Parasites

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Malaria infections remain a serious global health problem in the world, particularly among children and pregnant women in Sub-Saharan Africa. Moreover, malaria control and elimination is hampered by rapid development of resistance by the parasite and the vector to commonly used antimalarial drugs and insecticides, respectively. Therefore, vaccine-based strategies are sorely needed, including those designed to interrupt disease transmission. However, a prerequisite for such a vaccine strategy is the understanding of both the human and vector immune responses to parasite developmental stages involved in parasite transmission in both man and mosquito. Here, we review the naturally acquired humoral and cellular responses to sexual stages of the parasite while in the human host and the *Anopheles* vector. In addition, updates on current anti-gametocyte, anti-gamete, and anti-mosquito transmission blocking vaccines are given. We conclude with our views on some important future directions of research into *P. falciparum* sexual stage immunity relevant to the search for the most appropriate transmission-blocking vaccine.

Keywords: *Plasmodium falciparum*, gametocytes, humoral immunity, cellular immunity, mosquito immunity

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INTRODUCTION

Malaria is one of the most important parasitic infections with the highest burden of mortality and morbidity in sub-Saharan Africa. Despite progress and advances in the strategies to control the disease, malaria claimed the lives of approximately 445,000 people from among 216 million clinical cases globally in 2016; mostly in children under 5 years and pregnant women as reported by WHO (1). The increasing challenges posed by the emergence of resistance to antimalarials by malaria parasites and to insecticides by mosquitoes (2, 3) suggest the need for additional interventions aiming at transmission reduction such as vaccines. Moreover, targeting of multiple stages of the parasites might be the best strategy for any successful malaria vaccine (4), further highlighting the need for continuous identification and validation of alternative and effective targets.

Transmission blocking interventions either targeting gametocytes while in the human host or gametes in the mosquito are considered an essential part of malaria control strategies especially in the quest to eradicate malaria (5, 6). Malaria parasites (sporozoites) are transmitted through the bite of Anopheline mosquitoes. Once in the human system, the sporozoites migrate to the

liver where they undergo pre-erythrocytic multiplication (schizogony) leading to the production of merozoites that move into the bloodstream (erythrocytic stage; **Figure 1**). The pathology results from red blood cell (RBCs) invasion and further asexual replication of parasites within RBCs (erythrocytic schizogony) leading to massive RBC lysis, disrupted blood flow due to cytoadherence of parasite-infected RBCs to endothelial surfaces, anemia, and inflammation that may be lethal if untreated. Gametocytes are specialized stages of *Plasmodium* parasites that are essential for transmission from humans to mosquitoes. Initially, a certain proportion of the erythrocytic stage parasites undergoes a permanent differentiation also referred to as sexual commitment into both male (microgametocyte) and female (macrogametocyte) gametocytes (**Figure 1**). This process is known as gametocytogenesis (7, 8).

Sexually committed ring stage trophozoites from erythrocytic stages in peripheral circulation (9, 10) progress into gametocyte developmental stages 1 to IV while sequestered in bone marrow compartments (11–14). This constitutes the main reason why only late gametocyte stages are found in peripheral circulation. Early gametocytes are thought to sequester in tissues such as spleen and bone marrow through parasite-host interactions via parasite molecules less elucidated but probably PfEMP1, STEVORS, or RIFINS (14–16). The human host endothelial receptors mediating sequestration of developing gametocytes in the bone marrow and other organs however remain unidentified (17). Differentiation of male and female gametocytes occur during sexual commitment where the asexual precursor, schizont, give rise to either male or female gametocytes (7, 8).

After about 10–12 days of sequestered development, mature, male, and female gametocytes emerge and circulate in peripheral blood for a variable amount of time until taken up by mosquitoes (18, 19). Gametocytes do not replicate; however, hemoglobin digestion continues until they reach stage IV (20). In addition, gametocyte-specific mRNAs are produced and a subset of these, important for their stage development in the mosquito, are translationally repressed until gametocytes are taken up by the vector when they go back to peripheral circulation (21). The phenomenon governing the return of mature gametocytes in the peripheral blood is not clearly understood. Once ingested, gametocytes rapidly transform into male (microgamete) and female gametes (macrogamete) in response to environmental cues such as a rise in pH, reduction in temperature and exposure to xanthurenic acid (22). Exflagellation (male gamete induction) is followed by the expression of gamete-specific proteins (23). Fertilization of macrogametocytes by microgametes is preceded by 3 rounds of DNA replication by male gametocytes giving rise to 8 motile microgametes resulting in a zygote (**Figure 1**). The zygote elongates to form an ookinete which crosses the midgut wall to develop into an oocyst. Further cell divisions and development of the oocyst give rise to sporozoites. Following oocyst capsule rupture, thousands of sporozoites emerge and invade the mosquito salivary glands which then render the vector infectious to humans during a bloodmeal, thus completing the transmission cycle (24–26) (**Figure 1**).

The infectiousness and transmission potential of gametocytes is influenced by their prevalence and density (27), degree of maturity (28), and both mosquito and human immune responses (29, 30). Furthermore, the efficiency of transmission depends on the generation of sporozoites and therefore level of infectivity or sporozoite dose transmitted (31). Moreover, the sporogonic stages are exposed to the vector's natural immune responses (32–34). It should be pointed out that gametocyte infectiousness refers to the amount of mature gametocytes that can potently infect the mosquito (demonstrated by their ability to undergo further development) after ingestion whereas sporozoite infectivity refers to the dose of potent sporozoites capable of being transmitted to humans during subsequent blood meals.

Here, we review the available evidence for naturally acquired human immune responses against the sexual stages of *Plasmodium* parasites targeting gametocytes and gametes in human and mosquito hosts, respectively. The mosquito immune responses against the development of these sexual stages in the midgut are also discussed, and propositions are made for future research directions toward the design of appropriate transmission blocking vaccines.

NATURALLY ACQUIRED ANTIBODY RESPONSES TO GAMETOCYTE AND GAMETE ANTIGENS

For over three decades now there have been some efforts to illuminate antibody responses to gametocyte and gamete development in mosquitoes and their potential for transmission reducing immunity (TRI). TRI is based on observations of naturally acquired antibodies against gametocytes that are produced in the human host in response to proteins of gametocytes that were not taken up by mosquitoes (35). When these gametocytes die, they release intracellular proteins/antigens into the host circulation. Among these are proteins produced in gametocytes which are crucial for the extracellular parasite development in the mosquito midgut (36, 37). These antigens are then processed and presented by antigen presenting cells eventually eliciting humoral immune responses, which can cause substantial or complete blockade of parasite development (gametogenesis, fertilization) in the mosquito. This is the essence of TRI and forms the basis for the development of transmission-blocking vaccines (TBV). TRI occurs when human antibodies, taken up by a mosquito in a potentially infectious blood-meal containing male and female gametocytes, are able to prevent fertilization and/or development of ookinetes/oocyts/sporozoites in the mosquito and thus infection of the mosquito (38, 39).

Extensively studied antigens to date include gametocyte/gamete proteins such as Pfs230 and Pfs45/48 and the zygote/ookinete proteins Pfs25 and Pfs28 (37) also known as the TBV candidate (30, 37, 40–43). Anti-Pfs230 and Pfs48/45 antibodies target the so-called pre-fertilization phase while anti-Pfs25 and anti-Pfs28 antibodies represent the post-fertilization phases marked by the differences in the parasite stage and target antigens. As such parasite proteins are referred to as Pre- and post-fertilization antigens, respectively. Binding of these antibodies to their antigen either blocks their function

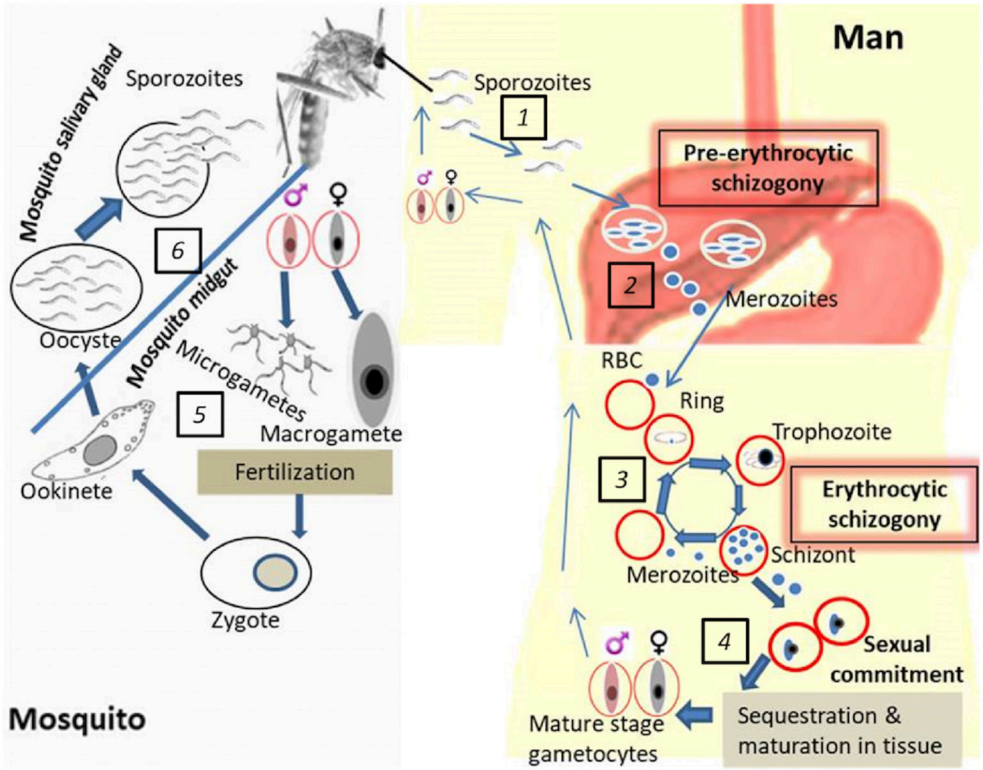

FIGURE 1 | Life cycle of *P. falciparum* development in the human host and mosquito vector. **(1)**. Mosquito's bite and release sporozoites into the human host followed by migration into the liver. **(2)**. Pre-erythrocytic schizogony: infection of hepatocytes and asexual multiplication of the parasites in the liver. **(3)**. Erythrocytic schizogony: translocation of parasites from the liver into the bloodstream accompanied by asexual multiplication and release of merozoites upon RBC rupture. **(4)**. Gametocyte generation: sexual commitment, sequestration of early gametocytes, maturation in tissues and release of mature gametocytes in blood (ready to be picked up by the vector). **(5)**. Parasite development in the mosquito midgut: exflagellation of male gametocytes prior to fertilization which yields the zygote which undergoes further development into a motile ookinete. **(6)**. Parasite development in the mosquito salivary gland: oocyst formation, sporozoite development, and release in the mosquito salivary gland (ready to be transmitted to the human host during subsequent mosquito bites).

essential for parasite development or facilitates complement-mediated gamete killing as shown for antibodies against Pfs230 (44).

Naturally occurring antibodies targeting Pfs230 and Pfs45/48 have been observed in field studies in The Gambia, Kenya, and Cameroon and were associated with reduced malaria transmission (30, 45). However, other studies reported that transmission reduction correlated with antibody responses to Pfs230 only (46) or with anti-Pfs48/45 antibodies only (6, 42, 43). These conflicting results may be due to differences in the history of exposure of study participants or existence of other co-infections.

A recent study by Stone et al. using field-based mosquito-feeding assays found mosquito infection rate to be significantly reduced for people harboring naturally acquired anti-Pfs48/45 and anti-Pfs230 antibodies. In addition, these antibodies were shown to be host gametocyte density-dependent and mechanistically associated with transmission reducing activity (TRA) (47, 48). In the same study, using protein microarray, 43 novel gametocyte proteins whose specific antibodies were associated with TRA were also identified (48). Among these 43 proteins, 16 predicted to be surface-expressed showed responses more similar to those of Pfs48/45 and Pfs230 in terms of TRA and as such warrant further investigations and characterization as TBV candidates (48, 49). However, the increase of natural seroprevalence to Pfs48/45 and Pfs230 with age found by Stone and colleagues did not corroborate a previous study by Ouedraogo et al. (50).

It is worth noting that antibodies against the post-fertilization antigens Pfs25 and Pfs28 have not been observed because these antigens are not exposed to the human immune system. If utilized in a vaccine, post-fertilization antibodies would not be boosted by natural malaria infections in vaccinated individuals. Nevertheless, antibodies against Pfs28 and Pfs25 have shown promise in blocking mosquito stage development and therefore transmission in *in vitro* experiments and are currently being evaluated in clinical trials (51).

The development and evaluation of antibody responses to all gametocyte/gamete-specific antigens and their effect on sexual stages in the mosquito face several challenges (51). First, evaluation of transmission reducing immunity relies heavily on mosquito feeding experiments, otherwise known as standard membrane feeding assays, where gametocyte-infected blood is fed to mosquitoes with or without antibodies to the

respective antigens. Dissection of mosquitoes 7 days after blood feeding for oocyt counts is used as an indirect measure of transmission blocking activity. These assays are time-consuming and labor-intensive. Second, it is not known what ingested antibody levels in the mosquito are required that would lead to a subsequent transmission blockade. The identification and validation of gametocyte surface antigens as vaccine candidates with transmission reducing activity (TRA) directly measurable in the human host will overcome these challenges, complement and strengthen current transmission blocking vaccine efforts (6, 52).

NATURALLY ACQUIRED ANTIBODY RESPONSES TO THE SURFACE OF GAMETOCYTE-INFECTED ERYTHROCYTES

It has been difficult to elucidate naturally acquired antibody responses to gametocyte-infected erythrocyte surface antigens (GSAs), distinct from those recognizing internally expressed gametocyte and gamete antigens, in natural human infections. This is largely due to the indirect effect of asexual stage immunity on the prevalence and density of gametocytes.

Naturally acquired sexual stage antibodies are known to be produced against *P. falciparum* gametocyte-infected erythrocyte surface antigens in human peripheral circulation (anti-gametocyte immunity) (4, 53). There are very few studies on human immune responses recognizing gametocyte-infected erythrocyte surface antigens referred to as anti- gametocyte immunity. This is in contrast to anti-gamete immunity which is raised against intracellular proteins of dead gametocytes which have some function at the gamete stages, or more broadly immune responses to gamete surface antigens (4, 6, 31, 52–54).

In the first investigation of its kind, plasma antibodies from gametocytemic Gambian children donated after antimalarial treatment were used to detect antigens on the surface of 3D7 cultured mature stage V gametocytes. Surprisingly, no antibody recognition of the surface of erythrocytes infected with developing gametocytes, stages I-IV, representing the stages known to be sequestered in deep tissues, were found (44, 54). In addition, children harboring these anti-GSA antibody responses were significantly less likely to carry gametocytes after subsequent infections suggesting an ability to control gametocytemia in these patients. It was also shown that malaria patient plasma samples with strong anti-GSA plasma antibody recognition of the mature gametocyte-infected erythrocyte surface were not more likely to recognize the surface of erythrocytes infected with asexual parasites and vice versa (54).

This was a proof of concept for the rationale to develop an anti-GSA transmission blocking vaccine. It derived its basis from epidemiological observations of specific immune suppression of gametocytes in Indonesia (53). *P. falciparum* gametocyte rates were reduced among semi-immune native Papuans, independent of immune control of asexual parasitemia, when compared to a transmigrant Javanese population with a history of lower malaria exposure. These findings suggest specific immune control of gametocytemia as the observations could not be explained by differences in the frequency or grade of parasitemia, illness or by known patterns of antimalarial treatment. Further, immunofluorescence tests with acetone-fixed whole gametocytes showed a correlation between antibody levels and reduced gametocytemia among the native Irianese (53).

The important observations by Saeed et al. (54), the ability of patient plasma to recognize GSA and the significant association with reduced gametocyte carriage, required further investigation. In order to rule out the fact that patient plasma antibody recognition of GSA on mature stage V gametocytes was not a deficiency or artifact of the 3D7 clone, we carried out recognition studies in plasma antibody samples from Ghanaian school children from a high endemicity region, against both a recent isolate and 3D7. In this study, plasma from asymptomatic school children collected over 5 sampling times at weekly intervals were tested against the surface of 3D7 mature gametocyte-infected erythrocytes as well as mature gametocytes derived from a 2012 clinical isolate of Kenyan origin, HL1204. Interestingly, we found plasma antibodies from all children bind to GSA of gametocytes derived from both clones to at least some extent. It was striking to note that plasma from Ghanaian children recognized the GSA on mature gametocytes of Kenyan origin, suggesting that perhaps the antigens detected might be conserved across geographical locations. Immature gametocytes from the clinical isolate were also tested against a selected number of plasma samples from Ghanaian children with strong anti-GSA antibody responses. Similar to the observations of Saeed et al. (54), no detectable recognition of GSA to asynchronous immature gametocytes was observed. These findings were corroborated by some gametocyte adhesion studies (15, 55), which posit that maturing gametocytes do not, as previously thought, sequester from peripheral circulation through adhesion to human bone marrow-derived endothelial surfaces and receptors (56–58). Nevertheless, further studies with tightly synchronized immature gametocyte preparations are required before we can rule out the possibility that developing gametocytes express adhesins involved in parasite ligand-host receptor interactions which mediates sequestration and elicit gametocyte-specific immunity (4).

To further test the prevalence of anti-GSA antibodies in the general endemic population, plasma donated by microscopically-confirmed parasite negative individuals were tested for antibody recognition to GSA. Forty-eight percent (24/50) of parasite-negative children and adults recognized the surface of mature gametocyte-infected erythrocytes (4, 59). Since submicroscopic gametocytemia could not be excluded, anti-GSA antibody carriage in cohort studies utilizing sensitive gametocyte detection methods such as RT-qPCR or QT-NASBA are needed to fully illuminate this relationship. Moreover, testing plasma donated from both gametocyte positive and negative children showed that our findings could possibly represent the general malaria-infected population. In addition, evidence was found that children who harbored anti-GSA antibodies were significantly but weakly associated with lower risk of gametocyte carriage (4). In addition, preliminary indirect evidence suggest that anti-GSA antibodies may be maintained over a period of time (4, 59).

CELLULAR IMMUNE RESPONSES TO GAMETOCYTES WHILE IN THE HUMAN HOST

Studies aiming at evaluating the cellular immune responses to the sexual stages compared to asexual ones of *Plasmodium* species are limited. However, there is evidence that such immunity exists. The transfer of T-cells from gamete-immunized mice was shown in the 1980s to markedly reduce gametocytemia in the recipient mice using the rodent species *P. yoelii nigeriensis* (60). Recipient mice also failed to effectively infect the mosquito vector *A. stephensi* as demonstrated by direct blood feeding (up to 95% transmission reduction) suggesting the direct impact of T-cells on transmission. However, the T-cell transfer had no effects on asexual stages (60). Good et al. (61) further showed that peripheral blood from non-exposed individuals contains T cells (clone) which proliferate and up-regulate interferon-gamma production upon stimulation with mature gametocyte-infected RBCs lysate. Similar results were also obtained in a hyper-endemic region in The Gambia by stimulation of peripheral blood mononuclear cells (PBMCs) of volunteers by gametocyte lysate (62). The detection of gametocyte-specific antibodies in the study participants by ELISA implied previous exposure to sexual stage parasites. Findings from this study suggest a T cell-dependent suppression of gametocytes or T cells helping B cells to fight against the malaria infection. Both asexual and sexual stage-specific antigens have equally been shown to elicit polyclonal T-cell responses in malaria non-exposed individuals (63). However, the reaction is not peculiar to gametocytes since it has been demonstrated that CD4 T Cells from non-exposed individuals react with PfEMP-1 via a Major Histocompatibility Complex (MHC) Class II-T cell receptor-independent Pathway (64). This could be associated to cross-reactivity from other infections. Whether this phenomenon is protective in children is not known.

In other studies, an increase in cytokine production such as TNF-α and IFN-γ was demonstrated in monkeys and humans infected with *P. cynomolgi* and *P. vivax,* respectively (65, 66). The increase in cytokine secretion correlated with the decrease in parasitemia and the inability of gametocytes to infect the mosquito vector and as such, cytokines and other PMBC-derived components (nitric oxide, antibodies) were believed to play a role in the loss of infectivity (66, 67). In their study in 1993, Naotunne et al. showed that the effect of PBMC-derived components on gametocyte infectivity was closely linked to the presence of white blood cells as no effect was seen in their absence (67). This negative effect on the infectivity of gametocytes appeared to be reversed in the presence of high concentration of an L-arginine analog (NGL-monomethyl arginine acetate) (68). This suggests that gametocyte inactivation in the presence of WBCs is achieved through an L-arginine-associated pathway mechanism. In the same line, an *in vitro* study conducted by Smith et al. (69) revealed that *P. falciparum* stage I and IIA gametocytes are to a large extent eliminated from the circulation by non-opsonic phagocytosis mediated by monocytes and macrophages. They showed through antibody inhibition assays and enzyme treatment that the interaction of PfEMP-1 and CD36 plays a major role in this innate defense against early gametocytes stages. This is in line with a previous study reporting the interaction of *P. falciparum* early gametocytes with CD36 receptor (70). A recent study conducted in India demonstrated a significant negative association between gametocytemia and IFN-γ in children (71).

Gametocyte-specific exoantigens (from gametocyte culture supernatants) have been shown to be able to stimulate the proliferation and activation of lymphocytes from *P. falciparum* exposed individual (72). In this study, T cell receptors gamma/delta (TCR γδ+), and $CD3^+$ $CD8^+$ and $CD3^+$ $CD4^-$ $CD8^-$ T cells were found to be up-regulated upon sensitization with these exoantigens. Particularly, the expression of the activation marker $CD25^+$ increased on stimulated $CD3^+$ and γδ T cells. The frequency of γδ T cells had previously been found to increase in the course of acute malaria (73). However, it is difficult to ascertain the specificity of the exoantigens because they could as well be coming from ruptured or dead asexual parasite iRBCs. As already indicated, there is a paucity of information on cellular immunity to gametocytes. Therefore, further investigations into the role of cellular immune responses to gametocytes and malaria transmission; and the identification/validation of the antigens involved, in a bid to contribute toward the development of an effective transmission blocking vaccine are required.

CELLULAR IMMUNITY TO SEXUAL STAGES WHILE IN THE MOSQUITO VECTOR

In the mosquito vector, killing of malaria parasites is not only mediated by vertebrate host-derived molecules but also by mosquito components as has previously been demonstrated (32–34). Studies have shown that only a small proportion of gametocytes ingested in the blood meal by the mosquito vector is transformed into oocysts and sporozoites; and only about 38% of mosquitoes that take gametocyte-containing blood become infected (32, 33, 74). This is largely due to the peritrophic membrane or matrix (PM) which constitutes a physical barrier to *Plasmodium species* and other pathogens (33, 75, 76). This membrane is formed after the ingestion of a potentially infectious blood meal by the mosquito and surrounds the ingested blood. It prevents direct contact between the pathogens in the blood and the midgut epithelium and by so doing interferes with midgut invasion (33, 76). However, ookinetes secrete the enzyme chitinase which destroys the chitineous PM and allows it to invade the midgut (33, 77). The midgut epithelial cells are also thought to secrete high amounts of nitric oxide synthase and peroxidases, which in turn leads to nitration of the gut epithelium with subsequent tagging of ookinetes for destruction by the complement system (33, 78, 79).

The innate immune response in the malaria mosquito vector is mediated mainly by hemocytes which eliminate pathogens such as bacteria, fungi, and protozoa by phagocytosis (80–82). The *Anopheles* species and other insects are known to have a complement C3-like protein called thioester-containing proteins (TEP) (80). TEP of *A. gambiae* (AgTEP1) has been shown to be valuable for the initiation of immune defense against *P. berghei*. TEP1 plays the role of opsonins and facilitates

the interaction between the parasite and the hemocytes with subsequent encapsulation, and killing of the parasite (80). Double knock-out of the TEP1 gene renders genetically selected refractory *Anopheles* strain susceptible to infection and increases the infectivity rates in susceptible *A. gambiae* (80). This vector defense mechanism has been shown recently to be by-passed by *P. falciparum* through its 6-cysteine protein P47-like (83). This protein is invaluable for *P. berghei* female gamete fertility (84) but in *P. falciparum*, it promotes the gametocyte-to-ookinete development and protects the ookinete from complement-dependent lysis (83).

In addition, infection of *A. gambiae* mosquito by ookinetes of *P. berghei* has been demonstrated to modulate the mosquito's immune system by up-regulating the expression of the antibacterial peptide defensin and a putative gram-negative bacteria-binding protein (85), and a TNF-α factor-like transcription factor (LL3) (86). Silencing of the LL3 gene was found to be associated with an increase in parasite survival, confirming its role in conferring mosquito resistance to the *Plasmodium* parasite. LL3 also affects the expression of another protein, a serine protease inhibitor (SRPN6), which equally confers resistance to invasion by *Plasmodium* (86).

Genomic and transcriptomic analyses of bacterial lipopolysaccharide-stimulated *A. gambiae* mosquitos revealed 23 immune-regulated genes which include putative protease inhibitors, serine proteases, and regulatory molecules (87). Interestingly, the protease inhibitor α-2-macroglobulin was found to be more specific in response to malaria parasite than bacterial infection as observed with mosquitoes fed on a *P. berghei*-infected hamster. This suggests that the immune response mounted by the mosquito vector may be pathogen specific, and other authors have reported similar findings (88). RNA gene interference (RNAi) experiments on *P. falciparum*- and *P. berghei*-infected *An. gambiae* revealed some common genes that confer resistance to both parasite species. However, other genes were found to exhibit species-specificity, conferring resistance only to one parasite species, namely a pattern recognition receptor (MD2-like receptor, AgMDL1) and an immunolectin, FBN39 for *P. falciparum* and the antimicrobial peptide gambicin and a novel putative short secreted peptide, IRSP5 for *P. berghei* (88). Together, these findings show that mosquitoes express molecules with anti-plasmodial properties which act as self-defense mechanism in the vector.

ANTI-GAMETOCYTE AND ANTI-GAMETE TRANSMISSION BLOCKING VACCINES

Up to date, it has been a difficult task developing an effective vaccine against malaria. This is due both to the complexity of the *Plasmodium* parasite life cycle and the polymorphic nature of its antigens (89, 90). However, the hope that an effective malaria vaccine is feasible is based on the observation that in endemic regions, clinically immune adults are protected from severe malaria and death compared to children (89). This could be attributed to the fact that natural immunity in adults is probably complex and dependent on immune responses to many stages. Interestingly, sera from immune individuals have been shown to inhibit gamete fertilization and development in the mosquito vector thereby interfering with disease transmission (91–93). This constitutes the basis of the development of malaria transmission blocking vaccines (TBVs). An emerging concept is to develop vaccines against antigens expressed solely in the mosquito's midgut to which the host immune system is not naturally exposed. Antibodies against those antigens from vaccinated individuals and animals have been shown to interfere with parasite viability and development in the mosquito midgut interaction (94–96).

As a limitation, TBVs are different from the other vaccine types (liver and blood stage vaccines) in the sense that they do not protect against disease in the vaccinees. However, they reduce the risk of transmission to other people by the mosquito vector and by so doing favor herd immunity; as such they have sometimes been referred to as altruistic vaccines (95). Two groups of target antigens (gene superfamilies) exist, namely pre-fertilization and post-fertilization antigens (**Table 1** and **Figure 2**) (48, 97, 107–111, 121). The list (**Table 1**) is not exhaustive both for pre- and post-fertilization antigens as some proteins remain unidentified to date. Some of these target antigens were characterized back in 1983 by Kaushal et al. (98), among them, Pfs48/45, Pfs47, Pfs230, and Pfs25 are immunogenic and less polymorphic, making them good vaccine candidates (122). Their use in combination with strong adjuvants or carrier proteins has been shown to boost their immunogenicity. Some of the adjuvants/carrier proteins used included Maltose Binding Protein (MBP)—Exoprotein A (EPA) from *P. aeruginosa*—Outer Membrane Protein Complex (OMPC)—modified Lickenase carrier (LiKM)—Virus-like particle (VLP)—Alhydrogel. Only two of these vaccine candidates, namely Pfs 230 and Pfs25, have entered clinical trial stage (123) and are reviewed in this paper. The potential of the other antigens (e.g., Pfs48/45) as TBV candidates has been recently reviewed by Chaturvedi et al. (122). Although Pfs 45/48 has not yet attained the clinical trial phase, previous studies demonstrated that antibodies against this antigen elicit up to 99% inhibition of oocyst intensity and 85% inhibition of oocyst prevalence (99). Hence the necessity to pursue studies with the Pfs 48/45 TBV candidate. Moreover, recent studies have identified new sexual stage antigens that require more attention (48, 121)

Pfs230 is a 363 kDa protein and a potent antigen of malaria TBV. It is a main component in the fertilization process as male gametes with impaired Pfs230 gene are incapable of interacting with red blood cells (RBCs) and forming exflagellation centers (100). This results in marked reduction in oocyst production and mosquito infectivity. Similar observations were made with genetically modified *P. falciparum* with a truncated chitinase 1 (PfCHT1) gene which could be due to the inability of affected ookinetes to invade the mosquito midgut (106). Administration of recombinant Pfs230 + Alhydrogel induced high titers of antibodies in rabbits which were found to have significant transmission reducing activity (101). It should be pointed out that only certain fragments of the recombinant Pfs230 antigen induce responses that lead to TRA (99). Significant associations between suppression of mosquito infectivity and anti-Pfs230

TABLE 1 | Sexual stage antigens in the human host and mosquito vector with transmission reducing activity/potentials.

Type	Antigen name	Function/role	References
Parasite pre-fertilization antigens	Pfs48/45	Male gamete attachment to female gamete	(6, 30, 42, 43, 97, 98)
	Pfs47	Fertilization process	(97, 98)
	Pfs230	Main component in the male fertilization process	(30, 41, 98–101)
	STEVORS	Sequestration of early gametocytes and deformability of mature gametocytes	(14–16)
	Plasmodium falciparum surface related antigen (PfSRA)	Erythrocyte invasion, unknown role in gametocytes	(102)
	Plasmodium falciparum LCCL domain-containing protein (CCP)	Parasite development in the mosquito	(103)
	Plasmodium falciparum CX3CL1-binding protein 2	Cytoadherence to host cells	(104)
	Plasmodium falciparum Gametocyte EXported Protein-5 (PfGEXP5)	Gametocyte switching	(105)
Parasite post-fertilization antigens	Pf25	Parasite survival and interactions with mosquito midgut	(37, 51, 98)
	Pfs28	Parasite survival and interactions with mosquito midgut	(37, 51, 98)
	Chitinase 1	Parasite invasion of the midgut	(33, 77, 106)
	Von Willebrand factor-A domain-related protein (WARP)	Ookinete attachment to the mosquito midgut, differentiation of ookinete to oocyst	(107)
	Circumsporozoite and thrombospondin-related anonymous protein (CTRP)	Transition from ookinetes into oocysts in the vector	(108)
	Membrane-attack ookinete protein (MAOP)	Ookinete midgut invasion in vector	(109)
	Secreted ookinete adhesive protein (SOAP)	Ookinete midgut invasion and oocyst development	(110)
	Cell-traversal protein for ookinetes and sporozoites (CelTOS)	Establishment of malaria infections in both vector and vertebrate hosts	(111)
Vector antigens	Midgut-specific alanyl aminopeptidase (AnAPN1)	Ookinete midgut invasion in vector	(75, 112–115)
	Carboxypeptidase B1	Parasite development in the vector	(116, 117)
	Serine protease inhibitors (serpins)	Regulation of the vector innate immune responses	(118)
	Saglin proteins	Vector salivary gland invasion	(119, 120)

antibody levels were also found using membrane feeding assays with sera collected from African populations (43, 45, 46, 124) and in mice (99). This vaccine candidate (125) has entered a phase 1 clinical trial in which the Safety and Immunogenicity of Pfs230D1M-EPA/Alhydrogel is being evaluated in adults in the US and Mali (data unpublished, https://clinicaltrials.gov/ct2/show/NCT02334462).

Pf25 is relevant for parasite survival and interactions with mosquito midgut molecules prior to invasion. Anti-Pf25 antibodies have been shown to halt parasite growth within the mosquito in membrane feeding assays as reviewed by Chaturvedi et al. (122). This vaccine candidate has undergone clinical trial phase 1 in combination with different carriers and adjuvants. Administration of Pf25-Viral-like-particles plus Alhydrogel® to mice resulted in high antibody titers with 100% transmission reducing activity (TRA) throughout the study (126). A clinical trial phase 1a with this combination was recently carried out in the United States (https://clinicaltrials.gov/ct2/show/NCT02013687). It appeared that the combination is safe with no serious adverse reactions observed in healthy volunteers even when higher doses are administered. However, the antibodies generated showed low TRA hence the necessity to prioritize vaccine adjuvant formulations for further investigations (127).

Another combination of Pfs25 and EPA (Pseudomonas aeruginosa ExoProtein A) plus Alhydrogel® was also demonstrated to be well tolerated by naïve individuals after several doses in a phase 1a dose-response clinical trial in the US which correlated with antibody titers (128). A Phase 1b trial of Pfs25-EPA/Alhydrogel® is currently ongoing in Malian adults (122). New promising multimeric Pf25-based Vaccine Candidates ChAd63 Pfs25-IMX313 and MVA Pfs25-IMX313 have recently been developed with promising results (129) and are now undergoing clinical trial phase 1a in the UK. These vaccine candidates consist of attenuated viruses (ChAd63-chimpanzee adenovirus 63 and MVA- modified vaccinia Ankara) encoding the parasite protein Pf25, which are fused to a carrier protein (IMX313-multimerization technology) as adjuvant (NCT02532049). In mouse models, Chad63Pfs25-IMX313 was safe and significantly more immunogenic with higher TRA than monomeric Pfs25 (130). Similar results were obtained with the *P. vivax* antigen (Pvs25H/Alhydrogel), and the *P. falciparum* ortholog Pfs25 in a mouse model. Anti-Pvs25H antibody

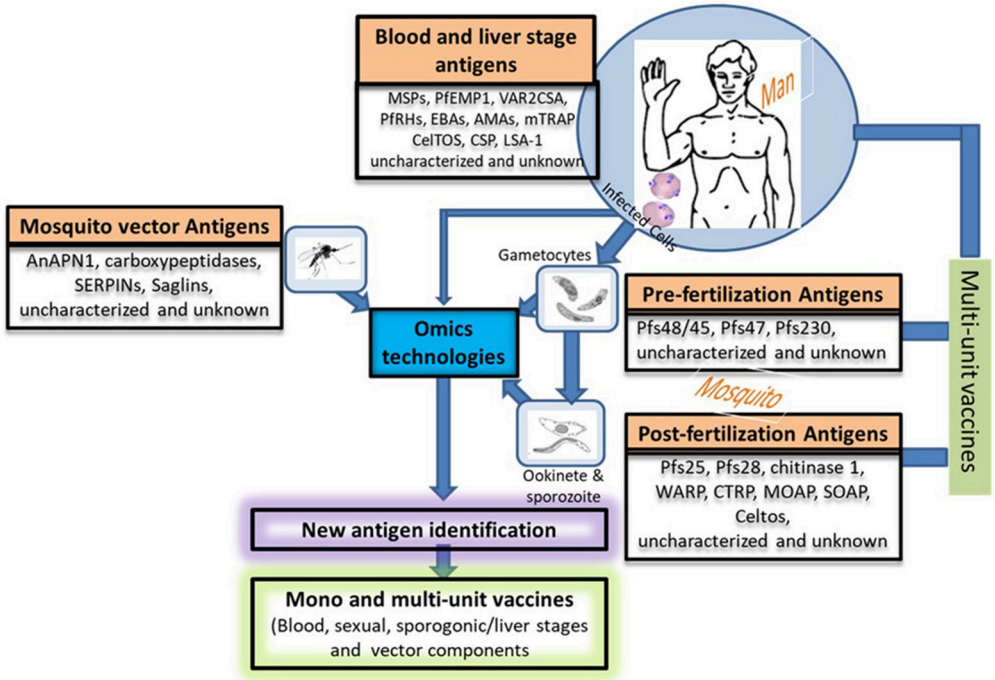

FIGURE 2 | Potential approaches for new vaccine candidate identification and vaccine development. This figure describes in a nutshell the life cycle of the *Plasmodium* species providing different stage-specific antigens and the strategies that could be used to develop more efficacious vaccine by combining potential vaccine candidates from various stages (host's and vector's). Uncharacterized and unknown antigens refer to proteins of unknown functions or proteins that have only been partially characterized such as the multigene families (*var, rif, and stevor*). The function of the proteins encoded by these multigene families have been tremendously studied in the asexual stages of *Plasmodium* but poorly exploited in their sexual counterparts. We propose that more attention should be paid to the sexual stages in terms of vaccine candidate identification and characterization. Nowadays, this could easily be done with the advent of Omics technologies. PfEMP-1, *Plasmodium falciparum* erythrocyte membrane protein-1; RIFINs, repetitive interspersed families of polypeptides; STEVORs, Subtelomeric variable open reading frame polypeptides; MSPs, Merozoite surface proteins; VAR2CSA, A variant of PfEMP-1 with high affinity to placental tissues; PfRHs, *Plasmodium falciparum* reticulocyte binding protein homologs; EBAs, Erythrocyte binding antigens; AMAs, Apical membrane antigens; CSP, Circumsporozoite protein; LSA-1, Liver-Stage Antigen; mTRAP, Merozoite thrombospondin-related anonymous protein; Pfs (230, 48/45, 25,28), *Plasmodium falciparum* gamete surface proteins (molecular weight in dalton); WARP, von Willebrand factor-A domain-related protein; CTRP, Circumsporozoite and TRAP-related protein; MAOP, Membrane-attack ookinete protein; SOAP, Secreted ookinete adhesive protein; CelTos, Cell-traversal protein for ookinetes and sporozoites; AnAPN1, *An. Gambiae* alanyl aminopeptidase; serpins, Serine protease inhibitors; *saglin* proteins.

levels peaked after the third vaccination and vaccine-induced antibodies were functional, giving significant TRA (96). This combination has proven to be safe in humans in a clinical trial phase 1 with similar immunogenicity and TRA as previously shown in mice (131).

The main limitations of these sexual stage vaccine candidates have been the systemic reactogenicity observed in some clinical trials, short-lasting antibody responses owing to the fact that the host has no or limited exposure to the antigens requiring multiple boosting doses and strong adjuvants. In addition the recombinant antigens are difficult to express in their native form (122). To circumvent the issue of limited exposure of pre-fertilization and post-fertilization antigens to the human immune system, it might be good to develop DNA or viral vector-based vaccines containing different antigens (132, 133). This ensures continuous production of the antigens of interest in the host hence permanent stimulation of the immune system, thus bypassing the necessity for multiple immunizations (132, 133). However, the main problem is that the attenuated virus used can revert and cause infections, it can also get integrated in the vaccinee's genome and lead to unforeseen consequences with the associated ethical issues. It would be valuable to make use of high class adjuvants such as the Polymeric nanoparticle- and microparticle-based adjuvant systems that ensure long term delivery of the antigen to the host system when administered (134). It would be good also to include some blood and liver stage antigens to such combinations so that the vaccinee benefits from the process (**Figure 2**).

ANTI-MOSQUITO TRANSMISSION BLOCKING VACCINES

Some mosquito components are invaluable for the sporogonic development of malaria parasites as they are involved in parasite invasion through interaction with parasite receptors. Antibodies raised against these components could be very useful in blocking parasite development in the mosquito vector (95). Thus, these components constitute another class of TBV candidates as their inhibition would likely minimize the risk of new infection in the

community (135). An alternative transmission blocking vaccine strategy could be to interfere with the interactions between the parasite and the midgut molecules of the mosquito vector which will lead to the inhibition of ookinete invasion and the development of mosquito stages. Many of such molecules have been identified and characterized (**Table 1** and **Figure 2**). These molecules have been shown to be more conserved than the parasite antigens and are immunogenic in non-human primates (75, 112–115).

Midgut-specific alanyl aminopeptidase (AnAPN1) is a Glycosylphosphatidyl inositol (GPI)-anchored antigen which plays a valuable role in ookinete invasion in *A. gambiae* as previously shown by Dinglasan et al. (113). Administration of a recombinant fragment of rAnAPN160–195 with Alhydrogel has been demonstrated to stimulate a sustained production of antibodies with transmission blocking activities as revealed by membrane feeding assays (112). The TRA was dose dependent with higher antibody levels attaining 100% efficacy, and functional in both the chromosomal M and S forms of *A. gambiae* vectors. Moreover, the *P. falciparum*-infected blood samples used for membrane feeding assays were collected directly from gametocyte positive individuals and the results obtained exceeded those that had been reported previously with laboratory strains (113). More importantly, anti rAnAPN160–195 antibodies had effects on both *P. falciparum* and *P. vivax* and based on that evidence the AnAPN1 TBV has been recommended for phase I clinical trials (112). This antigen is immunogenic in mice and rabbits even in the absence of adjuvants (113, 115). However, it is worth mentioning that another study by Kapulu et al. failed to replicate the finding reported here. In their study, anti-AgAPN1 IgG had no significant impact on oocyst prevalence (99). Antibodies to another GPI-anchored vector midgut protein, α-AgSGU, were also confirmed to have an effect on *P. falciparum* and *P. vivax* development in *An. gambiae* and *An. Dirus* (114). However, high doses of α-AgSGU antibodies were required to achieve 80% TRA rendering *α-AgSGU* less promising as a TBV target.

In the same line, the midgut carboxypeptidase gene of *A. gambiae* (cpbAg) has been shown to be up-regulated following *P. falciparum* gametocyte ingestion by the vector (116). In addition, anti-CPBAG antibodies were shown to inhibit the development of both *P. falciparum and P. berghei* in the vector's midgut. Antibodies directed against CPBA have also been demonstrated to be vector-unspecific in the sense that they also inhibit the development of the *P. falciparum* gametocytes in *A. stephensi* mosquitoes, which is the main malaria vector in Iran and neighboring countries (117). This confirmed the conserved nature of molecules across different vector as predicted using genomic and proteomic approaches (117) and implies that a vaccine designed with CPBA could provide cross-species protection. Thus, CPBA constitutes another promising TBV candidate.

The interaction between the *Plasmodium* sporozoite Thrombospondin Related Anonymous Protein (TRAP) and the mosquito Saglin proteins is a prerequisite for vector salivary gland invasion (119). This has been confirmed by *in vivo* down regulation experiments of *saglin* gene expression which revealed a negative association with salivary gland invasion (119). Moreover, *in silico* analysis of *saglin* revealed the presence of a signal peptide suggesting that it may be a secreted protein. If verified *in vitro* and *in vivo*, Saglin proteins could constitute a new promising candidate for TBV design (120). Similarly, RNA interference silencing and knock-down experiments have demonstrated the essentiality of the serine protease inhibitors (serpins) in the survival of *An. gambiae* and *An. stephensi* as well as in the development of parasites (*P. berghei*) within these vectors (136, 137). Serpins are regulators of the vector innate immune responses and they are involved in the clearance of protozoan parasites (137). Antibodies raised against the *An. gambiae* serpin-2 (AgSRPN2) have been shown to be *P. berghei*-specific in *An. gambiae* and *An. stephensi* as they failed to interfere with *P. falciparum* oocyst formation (118). This study demonstrated that mosquito innate immune response-related molecules could be used as targets for TBV design; however, further investigations are needed to identify and/or validate the right antigens. A limitation here will be that all of these proteins are likely to suffer from the same problems as gamete/ookinete antigens in the sense that several booster doses are required and antibodies may be short-lived.

FUTURE DIRECTIONS IN SEXUAL STAGE IMMUNITY AND VACCINE DEVELOPMENT

Apart from studies reported in these reviews (60–62, 69, 72, 73), studies on host cellular and humoral immunity to gametocytes are scarce if not inexistent. Given promising results in clinical trials of TBV experimental vaccines for malaria eradication, the antigens involved should be characterized further to explore their suitability as vaccine candidates. The generation of long-lived antibodies depends on the generation of long-lived plasma cells and memory B cells (MBCs) within germinal centers (GCs) of secondary lymphoid organs (138). The prerequisite for plasma cells and MBCs is the interaction between follicular T helper cells and B cells. Further investigations of these cell types vis-à-vis the identified antigens in the context of malaria infections are needed. Similarly, further studies aiming at identifying new antigens (mainly vector-, gametocyte-, ookinete-, and/or oocyste-related) using genomics, transcriptomics and proteomics approaches, the Sanger center parasite gene knock-out library and other bioinformatics strategies are warranted (139–141) (**Figure 2**). These approaches take advantage of next generation sequencing (NGS) and the availability of growing numbers of *P. falciparum* whole genome sequences to identify new antigens (142). These methods are relatively fast and high throughput, leading to the identification of a plethora of essential genes or antigens through comparative analyses (139, 141–143). The implications of omics in the fight against infectious disease was recently reviewed by Bah et al. (144). But this would be strengthened by the concomitant ability to cultivate the sequenced lines and generate sexual stages from them for phenotypic studies. Bioinformatic strategies can also overcome some of the difficulties in studying parasites such as *Plasmodium spp*. or *Trypanosoma spp*. which are genetically

diverse (140, 145–147). In addition, computer-based algorithms have been developed to delineate T-cell epitopes on essential parasite proteins directly from genome sequence data (148–150). Vaccine developers should consider designing multi-unit or multi-stage TBVs with components from both the parasite (precisely gametocyte antigens) and the vector as this will broaden their spectrum of action.

As far as the search for a vaccine is concerned, much has been done to understand the multigene family PfEMP-1. However, the other multigene family proteins such as RIFIN and STEVOR also constitute an important class of parasite molecules that deserve attention. These form part of the uncharacterized or partially characterized parasite antigen repertoire with respect to the sexual stages (**Figure 2**). The *stevor* multicopy family is made up of a set of 39 genes with 2–3 copies expressed at a time (151) while about 150–200 genes code for RIFINs with many copies expressed at a time as well (152). STEVOR and RIFIN proteins were recently shown to be implicated in rosetting which is a phenomenon associated with sequestration and clinical complications of the malaria (153–157). STEVORs are suspected to be implicated in the sequestration of early gametocytes in tissues such as the bone marrow and spleen as well as the deformability of the mature gametocytes (14–16). There is also evidence that STEVORs alter RBC membrane rigidity since RBC deformability has been shown to be linked to STEVOR dissociation from the mature gametocyte-infected RBC membrane (15). This implies that inhibiting the functions of STEVORS might negatively affect the development of gametocytes and by so-doing will also reduce disease transmission due to a reduced number of sexual stages being ingested by the mosquito vector during its blood meal. Despite their variable nature, the putative role of STEVORs in gametocyte development and sequestration certainly make this family a possible new class of TBV vaccine targets. Humoral responses to these antigens have been demonstrated (158). We therefore recommend further characterization (both humoral and cellular) of anti-STEVOR immune response, in the hope of finding additional clues in the search for efficient TBVs. However, the most pressing task is to develop further STEVOR-specific reagents demonstrating their relevance in anti-gametocyte immune responses and therefore transmission reducing immunity/activities.

In addition to STEVORS, many other Plasmodium antigens such as LCCL domain-containing protein family (103), *Plasmodium falciparum* Surface Related Antigen (PfSRA) (102), CX3CL1-binding protein 2 (104), Gametocyte EXported Protein-5 (PfGEXP5) (105) have been described as potential TBV candidates and as such deserve further characterizations.

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JK-O and BD designed and drafted the manuscript. CS, FB, GA, and BU reviewed and edited the manuscript. All authors approved the final version of manuscript for publication.

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REVIEW
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The Role of IL-10 in Malaria: A Double Edged Sword

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IL-10 produced by $CD4^+$ T cells suppresses inflammation by inhibiting T cell functions and the upstream activities of antigen presenting cells (APCs). IL-10 was first identified in Th2 cells, but has since been described in IFNγ-producing $Tbet^+$ Th1, $FoxP3^+$ $CD4^+$ regulatory T (Treg) and IL-17-producing $CD4^+$ T (Th17) cells, as well as many innate and innate-like immune cell populations. IL-10 production by Th1 cells has emerged as an important mechanism to dampen inflammation in the face of intractable infection, including in African children with malaria. However, although these type I regulatory T (Tr1) cells protect tissue from inflammation, they may also promote disease by suppressing Th1 cell-mediated immunity, thereby allowing infection to persist. IL-10 produced by other immune cells during malaria can also influence disease outcome, but the full impact of this IL-10 production is still unclear. Together, the actions of this potent anti-inflammatory cytokine along with other immunoregulatory mechanisms that emerge following *Plasmodium* infection represent a potential hurdle for the development of immunity against malaria, whether naturally acquired or vaccine-induced. Recent advances in understanding how IL-10 production is initiated and regulated have revealed new opportunities for manipulating IL-10 for therapeutic advantage. In this review, we will summarize our current knowledge about IL-10 production during malaria and discuss its impact on disease outcome. We will highlight recent advances in our understanding about how IL-10 production by specific immune cell subsets is regulated and consider how this knowledge may be used in drug delivery and vaccination strategies to help eliminate malaria.

Keywords: IL-10, malaria, protozoan, T cells, Inflammation

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INTRODUCTION

Malaria remains a major public health problem in tropical and sub-tropical regions of the world despite substantial efforts to reduce associated morbidity and mortality. There are still around 250 million cases and 500,000 deaths annually, with young children in sub-Saharan Africa being most affected (1). A major unmet medical need for malaria is an effective vaccine. No vaccine tested to date in malaria endemic areas has performed as well as when tested in healthy volunteers, including RTS,S/AS01 (2–4).

$CD4^+$ T cells play critical roles in coordinating immune responses during infection by differentiating into functional subsets best suited to control pathogen growth (5). Diseases caused by intracellular protozoan parasites, such as *Plasmodium* species, require the generation of IFNγ-producing, $Tbet^+$ $CD4^+$ (Th1) cells to promote antigen capture and presentation by dendritic cells (DCs) and macrophages, as well as stimulate phagocytic cells to kill captured

or resident pathogens (6). However, the inflammatory cytokines produced by Th1 cells can also damage tissues. In addition, recent data suggests that Th1 cell development may also influence the development of T follicular helper (Tfh) cells, another important $CD4^+$ T cell subset in malaria needed for the expansion of antigen-specific B cell populations and the production anti-parasitic antibody (7, 8). Hence, a better understanding about the development of $CD4^+$ T cell responses during malaria is needed to improve strategies aimed at improving anti-parasitic immunity.

The development of a robust host immune response is essential to eliminate parasites that cause malaria and protect against re-infection. Concurrently, these responses need to be tightly regulated to avoid immune-mediated damage to host tissue. This requires the establishment of immunoregulatory networks which ultimately determine the magnitude of immune response following infection. However, if these networks over-power anti-parasitic immunity too early, parasites can persist and cause associated disease. Many molecules and cell types contribute to these immunoregulatory networks, including anti-inflammatory cytokines such as interleukine-10 (IL-10) and transforming growth factor (TGF)β, immune check point molecules such as PD-1, CTLA-4, and LAG-3, as well as $CD4^+$ $FoxP3^+$ regulatory T (Treg) cells. However, our understanding about how immunoregulatory networks develop following *Plasmodium* infection and are maintained after resolution of infection is still incomplete.

One possible explanation for the failure of RTS,S/AS01 vaccine is the early imprinting of potent, pathogen-specific immunoregulatory networks in children following first exposure to malaria that prevents the generation of robust, vaccine-induced anti-parasitic immunity (9). Hence, targeting these networks may be critical step needed for malaria vaccines to stimulate long-lasting, anti-parasitic immunity in disease-endemic areas.

IL-10 has emerged as an important regulatory molecule in malaria that protects tissues by preventing excessive inflammation (10). It suppresses inflammation not only by directly dampening pro-inflammatory cytokine and/or chemokine production, but also by down-regulating the expression of MHC-II and co-stimulatory molecules on antigen presenting cells (APCs) and increasing expression of immune checkpoint molecules (11–13). IL-10 is secreted by many different cells, including B cells, Th1, Th2, Th17, and Treg cells, as well as innate immune cells such as macrophages and DCs (14). More recently, IL-10-producing Th1 (type 1 regulatory; Tr1) cells were found to develop relatively quickly in healthy volunteers participating in controlled human malaria infection (CHMI) studies and children living in malaria-endemic areas (15–18). Results from both pre-clinical malaria models and human studies show that IL-10 not only protects against severe disease, but also inhibits protective anti-parasitic immunity. In this review, we will discuss the role of IL-10 during the blood stage of experimental and human malaria, as well as describe the cellular sources of IL-10 and how the production of this potent anti-inflammatory cytokine is regulated. We will also examine how IL-10 mediated immune response may be manipulated to improve vaccine efficacy and/or current drug treatment regimes.

IL-10 AND MALARIA

The balance between host pro- and anti-inflammatory immune responses plays a critical role in determining the outcome of *Plasmodium* infection. A weak pro-inflammatory response may result in uncontrolled replication of parasites, while an excessive pro-inflammatory response may cause tissue damage, such as occurs in severe malaria syndromes, including cerebral malaria and multi-organ failure. Studies from mice have identified a clear role of IL-10 in controlling inflammatory responses and preventing tissue damage (14). IL-10-deficient mice infected with *P. chaubadi chaubadi* AS displayed exacerbated disease pathology, including hypoglycemia, hypothermia, and a loss in body weight, along with enhanced pro-inflammatory cytokine (IFN-γ, TNF-α, and IL-12) production (19). The excessive pro-inflammatory conditions in *P. chabaudi* AS-infected IL-10-deficient mice were also thought to cause parasite sequestration to the brain, associated with cerebral edema and hemorrhages (20). IL-10 has also been reported to play a protective role in experimental cerebral malaria (ECM) caused by *P. berghei* ANKA (21). In this model, decreased expression of IL-10 mRNA in spleen and brain tissue was associated with susceptibility to ECM. Sequestration of parasitized RBC (pRBC) in brain was mediated by ICAM-1 expressed by endothelial cells, and IL-10 inhibited expression of ICAM-I on these cells, thus providing a potential mechanism for the prevention of pathology associated with ECM. In a lethal *P. yoelii* infection in mice, production of IL-10 and TGF-β were thought to inhibit pro-inflammatory responses, and this was correlated with high parasitemia and severe anemia (22, 23). IL-10 has also been reported to promote hyper-parasitemia in mice infected with *P. chabaudi adami* (24). Therefore, data from mouse models of malaria indicates that IL-10 is required to protect host tissue from inflammation, but by doing so, can also promote growth of parasites and associated disease manifestations.

In a prospective longitudinal study conducted in a malaria endemic area, *IL10* gene polymorphisms associated with high IL-10 production were found to increase the risk of developing clinical malaria in young children (25). High levels of circulating IL-10 have been reported in patients with mild, severe and cerebral malaria (26, 27). Similar to studies in pre-clinical models, African children with severe anemia had lower plasma IL-10 levels than patients with moderate anemia or cerebral malaria, suggesting that IL-10 plays an important role in preventing severe anemia (28). However, a case control study in an African population with mild or severe malaria showed that both IL-10 and TNF-α were elevated in severe malaria and positively correlated with parasitemia (29). In another study with children living in a holo-endemic area of Western Kenya, higher ratios of plasma IL-10 to TNF levels were strongly associated with protection against severe malaria anemia, providing evidence that IL-10 may be protective by inhibiting TNF activity (30). This was supported by data from pre-clinical malaria models that have

shown over-expression of TNF can suppress haematopoiesis in the bone marrow and promote RBC destruction (31), while IL-10 is thought to enhance hematopoietic activity (32). High levels of plasma TNF have also been associated with anemia and high-density *P. falciparum* infection in Zairian children (33), as well as being associated with other severe malaria complications such as renal failure (34). Thus, IL-10 appears to play a critical role in regulating the pathogenic effects of TNF during malaria, but in performing this important role, IL-10 may promote high-density infections that can result in other complications of malaria, including accumulation of pRBC in tissue that can cause hypoxia and direct damage to the vasculature.

IL-10 suppressed IL-12 production by monocytes (35), which was required for the development of protective immunity against malaria and skewing the cytokine production pattern toward a pro-inflammatory response (36, 37). A study in patients with severe malaria anemia living in a holo-endemic region of western Kenya showed that ingestion of *Plasmodium*-derived pigment (hemozoin; [PfHz]) by monocytes, suppressed IL-12 production in an IL-10-dependent manner (38). IFNγ signaling is critical for the development of hematopoietic progenitor subsets during acute experimental malaria (39), and given the important role for IL-12 in IFNγ production, reduced levels of IL-12 would likely impact hematopoiesis during malaria.

IL-10 can also augment antibody production and B cell maturation (37, 40). In experimental malaria caused by *P. yoelli* infection of C57BL/6 mice, B cell intrinsic IL-10 signaling enhanced germinal center (GC) B cell responses by limiting IFNγ activity and subsequent Tbet expression by these cells, thereby promoting antibody production and parasite clearance (41). Hence, IL-10 may be beneficial for the development of humoral immunity, but detrimental for cell-mediated immune responses during malaria.

CELLULAR SOURCES OF IL-10 DURING PLASMODIUM INFECTION

IL-10 production was initially identified in Th2 cells (42), but has since been shown to be produced by many immune cells, including Th1 cells (43, 44), Treg cells (45), IL-17-producing $CD4^+$ T (Th17) cells (46), Tfh cells (47), $CD8^+$ T cells (48), B cells (49), including regulatory B cells (50), NK cells (51), and γδ T cells (52, 53). Additionally, innate immune cells such as macrophages and DCs (12) have also been shown to produce IL-10. In both lethal and non-lethal mouse models caused by *P. yoelii* infection, the major source of IL-10 were FoxP3-negative $CD4^+$ T cells that didn't produce Th1, Th2, or Th17-associated cytokines, and these cells not only prevented hepatic immunopathology but also suppressed the effector T cell response, preventing parasite clearance (54). These regulatory cells are amongst several specialized $CD4^+$ T cell sub-populations which emerge from the thymus as conventional $CD4^+$ T cells and acquire regulatory functions in the periphery following exposure to inflammatory conditions (55–57). The most well-studied of these subsets are IL-10-producing Th1 (Tr1) cells that have been identified in many infectious diseases, including visceral leishmaniasis (58), tuberculosis (59), and human immunodeficiency virus (60). Importantly, they have also be identified as an important immunoregulatory cell population in African children with *P. falciparum* malaria (16–18, 61), as well as in healthy volunteers participating in CHMI studies (15). A high frequency of antigen-specific Tr1 cells were also found in neonates whose mothers had active placental malaria during pregnancy, suggesting that these cells might be able to influence anti-parasitic immunity from very early in life (62). Hence, the rapid generation of Tr1 cells during malaria may play a critical role in determining the outcome of infection. Furthermore, because these cells are likely to be generated prior to vaccination in malaria endemic areas, they are also likely to impact the efficacy of malaria vaccines, and their presence and function may be an important factor contributing to the failure of these vaccines to date.

γδ T cells play several different roles in host defense against *Plasmodium* infection (63), including their rapid expansion upon exposure to *P. falciparum* antigen in malaria naïve individuals (64). However, studies in children with chronic malaria exposure showed that the $V\delta2^+$ subset of γδ T cells declined in number and switched from a predominant pro-inflammatory response to an anti-inflammatory response that was postulated to contribute to clinical tolerance during malaria (52). Another recent study in a malaria endemic area found a subset of γδ T cells from uncomplicated malaria patients expressing Vδ9 T cell receptor that expanded and produced IFNγ and IL-10 when cultured in presence of *P. falciparum* antigen (53). Given that these γδ T cells produce IL-10 and can provide help to B cells for antibody production (65), they may play a role in the acquisition of natural immunity against malaria.

NK cells have also been reported to produce IL-10 during many systemic infections (66, 67). A recent study in mice infected with *P. berghei ANKA* showed that systemic inflammation during ECM stimulated NK cell IL-10 production but was induced too late to prevent inflammation-mediated disease pathology (51). However, in the same study, treatment of mice with recombinant IL-15 complexed with antibody to extend cytokine half-life and target NK cell induced IL-10 production which protected against disease without affecting the parasite burden.

REGULATION OF IL-10 BY CYTOKINES

Tr1 cells are an important immunoregulatory $CD4^+$ T cell subset that not only prevent immune pathology during *Plasmodium* infection but can also promote establishment of infection by suppressing Th1 cell-mediated anti-parasitic immunity. The manipulation of this regulatory T cell subset is being considered for a wide range of immunotherapeutic applications, including in organ transplantation, rheumatoid arthritis, colitis, and cancer (68–71). Therefore, a better understanding about how IL-10 production is initiated and maintained by Tr1 cells is required for the development of new therapeutic approaches targeting this cell population. Tr1 cells are $Tbet^+$ $Foxp3^-$ $CD4^+$ T cells that are most likely derived from Th1 cells, and acquire an ability to co-produce IL-10 and IFNγ under inflammatory conditions

(72). IL-27 has been identified as a critical regulator of T cell IL-10 production in humans and mice (46, 73–76). Generally, IL-27 produced by APC's, such as DC's and macrophages, can induce expression of the transcription factors c-Maf and aryl hydrocarbon receptor (Ahr) via STAT1 and STAT3-dependent mechanisms, which in turn, stimulated IL-21 production by $CD4^+$ T cells which acted in an autocrine manner to expand IL-10-producing Tr1 cells (77–79) (**Figure 1**). In mouse models of malaria, a critical role for IL-27 signaling in the regulation of pro-inflammatory Th1 cell responses and suppression of immune-mediated pathology has been reported (80–83). A study with *P. chaubadi* AS infection showed that although Treg cells produced IL-10 during infection, Tr1 cells were the important source of IL-10 for protection against severe immune-mediated pathology, and that although the generation of these Tr1 cells was dependent on IL-27 signaling, it was independent of IL-21 (84). Similarly, another study with mice infected with *P. yoelli* confirmed that Tr1 cell generation was dependent on IL-27 receptor signaling during blood stage malaria (85). Thus, IL-27 is a critical regulator of IL-10 producing Tr1 cells in mouse models of malaria. The involvement of IL-27 in Tr1 cell generation in human malaria has not been fully established, although patients with severe malaria were reported to have reduced IL-27 plasma levels, compared to uncomplicated malaria patients and endemic controls, suggesting a potential role in controlling pathology (86). Additionally, many of the intracellular signaling

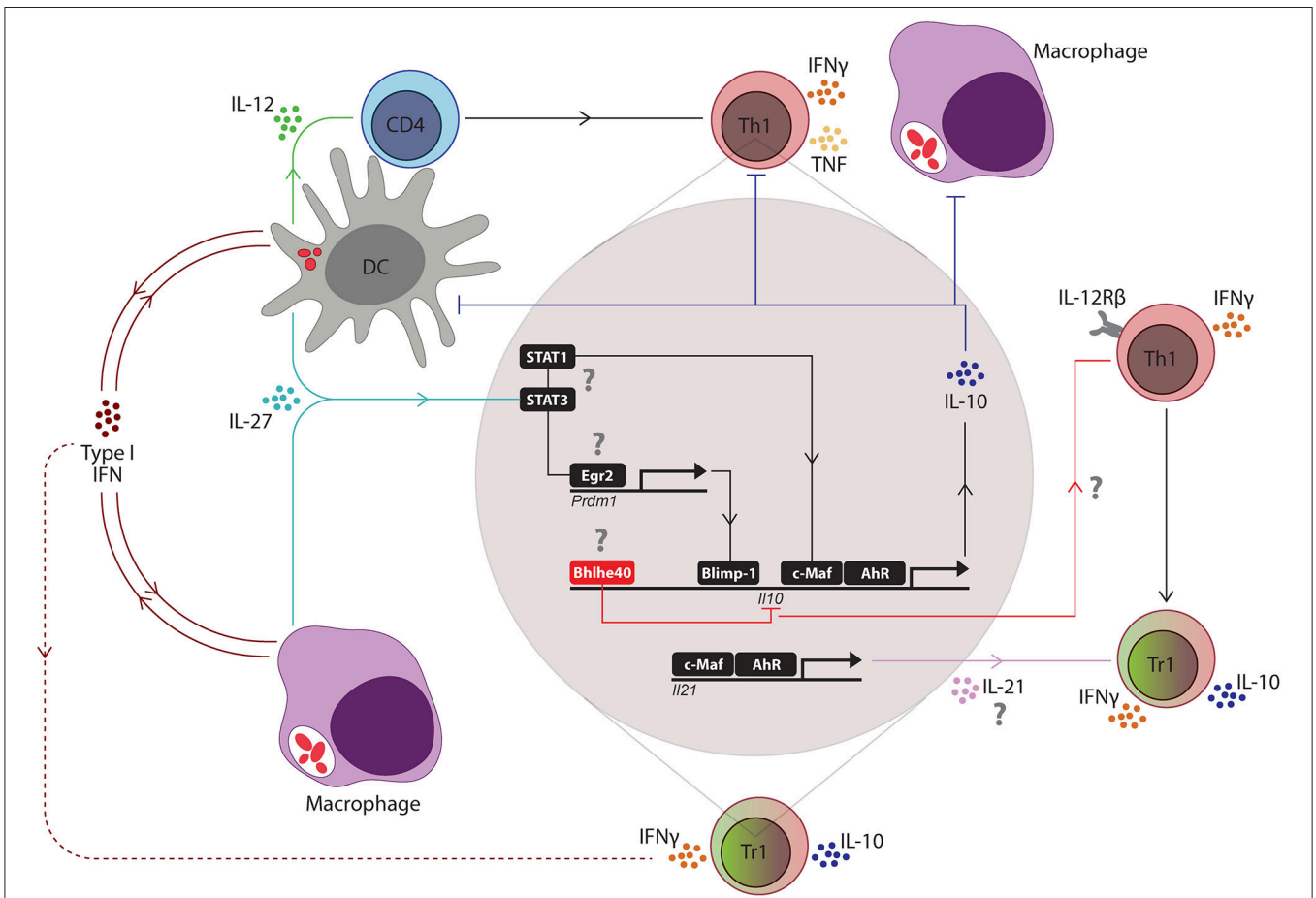

FIGURE 1 | Key regulators of the Th1/Tr1 cell axis in mouse malaria models**.** Following blood-stage infection with *Plasmodium* parasites, dendritic cells (DC's) recognize parasite molecules and produce IL-12 to drive the expansion of antigen-specific $CD4^+$ T cells into IFNγ-producing Th1 cells. Soon after, type I interferon (IFN) and IL-27 production is initiated by macrophages and DC's, presumably in response to activation of pattern recognition receptors by parasite molecules. Type I IFNs promote the development of IL-10-producing Th1 (Tr1) cells by T cell-independent and T cell-dependent activities (dashed red line), depending on the *Plasmodium* species. IL-27 stimulates STAT1 and STAT3-dependent transcription of c-Maf and aryl hydrocarbon receptor (AhR) in Th1 cells, which then drive *IL-10* and *IL-21* gene transcription. T cell receptor signaling of Th1 cells promotes expression of the transcription factor Erg2, which in turn, induces *Prdm1* (encoding Blimp1) transcription and IL-10-production by Th1 cells in a STAT3-dependent manner. IL-21 acts as an autocrine growth factor for IL-10-producing Th1 (Tr1) cells. IL-10 produced by these cells can suppress the activity of Th1 cells and phagocytes, as well as the antigen presenting capacity of DC's and macrophages. However, the Bhlhe40 transcription factor can also be upregulated in Th1 cells to block *IL-10* gene transcription and promote IFNγ production and IL-12 receptor β (IL-12Rβ) chain expression, which both re-enforce Th1 cell development and activity. Thus, Bhlhe40 has an important role in determining the balance between Th1 and Tr1 cell development. The small red ellipsoids in DC's and macrophages represent captured parasites and associated antigens. The question marks (?) indicate pathways not yet validated in *Plasmodium* infections.

pathways described above and below (**Figure 1**) have yet to be confirmed in samples from experimental malaria or malaria patients. This is clearly an important gap in our knowledge. It should also be noted that Tr1 cells are likely to represent a heterogenous cell population (87), and cytokine production and responsiveness may be regulated distinctly, depending on local immune conditions.

More recently, type I interferons (IFNs) have emerged as important regulators of IL-10 production by Tr1 cells. Type I IFNs comprise a large family of cytokines that includes several types of IFNα and two types of IFNβ proteins which all signal through the common IFNα receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains which signal via STAT-1 and STAT-2 (88, 89) to mediate diverse functions during many infections (90). Polymorphisms in the *IFNAR1* gene have been associated with increased risk of severe malaria in The Gambia (91, 92), while a whole-brain transcriptional analysis in genetically resistant and susceptible inbred mice infected with *P. berghei* ANKA identified type I IFN-dependent transcriptional program associated with the pathogenesis of severe malaria ECM (93). Type I IFNs suppress $CD4^+$ T cell-dependent parasite control during experimental blood-stage malaria by modulating the function of $CD8\alpha^-$ cDC following *P. berghei* ANKA infection, rather than acting directly on $CD4^+$ T cells (94, 95). Another study in mice infected with *P. yoelli* YM showed that type I IFNs produced by plasmacytoid DCs (pDCs) activated conventional DCs (cDCs) and macrophages for generating B and T cell responses which are required for controlling parasitemia and mortality during late phase of infection (96). However, another study in mice infected with *P. yoelli* showed that type I IFNs directly promoted the expansion of Tr1 cells (97) (**Figure 1**). Significantly, in CHMI studies, type I IFNs produced by several different cell sources were found to be important regulators of developing anti-parasitic immunity. Type I IFNs not only suppressed innate immune cell function and parasitic-specific $CD4^+$ T cell IFNγ production, but also promoted the development of parasitic-specific Tr1 cells (9). Thus, IL-27 is a major mediator of Tr1 cell development in mouse models of malaria, but to date, the main driver of Tr1 cell generation identified in humans infected with *P. falciparum* are type I IFNs. Whether these results point to separate pathways for Tr1 cell generation in humans and mice is still not clear, but if so, this has significant ramifications for developing strategies to modulate Tr1 cells for clinical advantage.

TNF is a potent pro-inflammatory cytokine that has been implicated in malaria pathogenesis. As mentioned above, the ratio of plasma IL-10 to TNF plays an important role in determining whether children with malaria develop anemia. It is often assumed that the relationship between these two cytokines is characterized by IL-10 dampening the pro-inflammatory activity of TNF. However, TNF can also promote IL-10 production, as demonstrated by TNF playing a major role in lipopolysaccharide-induced IL-10 secretion by human monocytes (98). Similarly, TGFβ was reported to play a protective role against severe malaria in mice (99), and TGFβ can drive IL-10 production by several different $CD4^+$ T cell subsets, including Treg, Th17 and other FoxP3-negative cells (100). Therefore, modulation of IL-10 production in malaria may be achieved by targeting the activities of upstream activating cytokines, such as IL-27, IL-21, type I IFNs, TNF, or TGFβ. However, as indicated previously, the precise roles for these cytokines in clinical malaria still needs to be fully elucidated before these strategies can be developed and implemented.

TRANSCRIPTIONAL REGULATION OF IL-10

As discussed earlier, IL-10 can be produced by most $CD4^+$ T cell subsets in various inflammatory settings, but whether IL-10 production is mediated by common transcription factors or cell lineage-specific transcription factors is unclear. Many transcription factors, including c-Maf, have been shown to modulate *IL10* gene expression *in vitro* (77, 101, 102). The ligand-activated transcription factor Aryl hydrocarbon Receptor (AhR) has also been shown to promote the development of Tr1 cells in humans (102). During IL-27-mediated Tr1 cell differentiation, AhR physically associates with c-Maf and trans-activates the *IL10* and *IL21* promoters (78) (**Figure 1**). A recent study in mice infected with *P. chabaudi* AS showed that c-Maf regulates T cell IL-10 production and T cell-specific c-Maf-deficiency was associated with greater acute-phase pathology, compared to control mice, but had little effect on blood parasitemia, similar to the phenotype observed in IL-10-deficient mice (103). Thus, although c-Maf-dependent T cell IL-10 production protected against the detrimental impact of inflammation, it had a minimal effect of the development of anti-parasitic immunity in this non-lethal, mouse malaria model.

Another transcriptional regulator, B lymphocyte induced maturation protein (BLIMP)1, has been shown to play an important role in IL-10 production by Treg cells (104). BLIMP1 is induced by IL-12 in a STAT4-dependent manner and was shown to control IL-10 expression by Tr1 cells in mice infected with *Toxoplasma gondii* (105). In chronic lymphocytic choriomeningitis viral infection, as well as in central nervous system-related autoimmunity, BLIMP1 was identified as a critical regulator of IL-10 production by Tr1 cells (106, 107). IL-27-dependent production of the early growth response gene 2 (Egr2), a transcription factor required for T cell anergy induction, was also required for IL-10 production by Tr1 cells in a BLIMP1-dependent manner (108, 109). BLIMP1-mediated IL-10 production by Tr1 cells was recently reported in experimental malaria (97), and BLIMP1-dependent IL-10 production by Tr1 cells protected against IFNγ-dependent, TNF-mediated splenic tissue damage, but also limited the control of *P. chabaudi* AS blood parasitemia (110).

More recently, basic helix-loop-helix family member e40 (Bhlhe40) has been identified as a negative transcriptional regulator of IL-10 production during *Mycobacterium tuberculosis* (111) and *T. gondii* infection (112). Interestingly, Bhlhe40 regulated IL-10 production in both T cells and DCs during *M. tuberculosis* infection by binding directly to the *Il10* gene promoter in both cell populations (111). In the case of *T. gondii* infection, Bhlhe40 promoted Tbet-dependent IFNγ production

by $CD4^+$ T cells, while also suppressing IL-10 production in this cell population (112). Given the many similar mechanisms of T cell IL-10 generation between these two infectious diseases and malaria, it will be important to establish the role of this transcription factor in IL-10 production during *Plasmodium* infections. Another basic helix-loop-helix family member Twist-1 also regulates IL-10 production (113), although little is currently known about its role in infectious diseases settings.

Therefore, key transcriptional regulators of IL-10 production have been identified with potentially important roles in malaria (**Figure 1**), although as mentioned previously, many still need to be validated with samples from pre-clinical models of malaria or malaria patients. However, this area of research is still at an early stage and it will be necessary to carefully elucidate the specific cell populations in which these transcription factors operate, the range of genes they regulate and whether their modulation can change IL-10 activity to improve anti-parasitic immunity without causing tissue damage.

THERAPEUTIC MANIPULATION OF IL-10

IL-10 plays a critical role in the immunoregulatory networks that protect tissue from infection-mediated inflammation during malaria, and there is convincing mechanistic evidence form pre-clinical malaria models and associative data from malaria patient samples showing key roles for IL-10 is preventing several severe manifestations of malaria, including the development of anemia and damage to organs. These actions of IL-10 are like a double-edged sword, cutting both ways, as they can both suppress important anti-parasitic immune responses, and in particular, the functions of Th1 cell responses, but also protect the host from tissue damage. Furthermore, as mentioned above, IL-10 may also promote anti-parasitic antibody production by B cells. Therefore, given these beneficial and detrimental roles for IL-10 during malaria, it is critically important that we improve our understanding about how IL-10 production is regulated and the specific roles for IL-10 produced by different cell populations. We know that parasite-specific Tr1 cells develop early in children living in malaria endemic areas, as well as in healthy volunteers participating in CHMI studies with *P. falciparum*. However, given the important roles these cells play in preventing disease pathology, as outlined above, modulation of their activity to improve vaccine efficacy or the development of immunity after anti-parasitic drug treatment may be dangerous. However, if modulation of IL-10 production by specific cell populations or subsets can be achieved, leaving in place mechanisms to protect tissue from inflammatory mediators, then clinical benefits may be achieved. This will require the identification of unique cellular signaling pathways that regulate IL-10 production in different cell populations. In addition, strategies to target specific cell signaling pathways will need to be developed.

CONCLUDING REMARKS

IL-10 is a critical immunoregulatory molecule with both positive and negative roles during malaria. Blocking IL-10 activity may promote anti-parasitic immunity by enhancing APC functions and associated T cell activity. However, this will likely result in concomitant tissue pathology and related disease. Therefore, a more selective strategy for IL-10 modulation will be needed. As we identify the cell subsets producing this cytokine, and learn more about upstream regulators, the cell signaling pathways, transcription factors and post-translational modifications controlling IL-10 production, we may be able to manipulate IL-10 activity to improve anti-parasitic immunity in response to vaccination or drug treatment. Although IL-10 is a promising target for immune modulation, it is not the only such target for trying to improve outcomes in malaria. Immune checkpoint molecules, other anti-inflammatory cytokines and alternative host-directed therapies have also been identified (9, 57, 114). Ideally, different approaches should be tested in parallel to establish the safest and best approach to take. However, robust pre-clinical and clinical models will be required, as well as appropriate resourcing. The development of effective, long-lasting immunity to malaria through vaccination or drug-mediated strategies is an important priority, and our increasing knowledge should help make this possible.

AUTHOR CONTRIBUTIONS

RK and CE researched the work and wrote the paper. SN prepared the figure. All authors reviewed and edited the manuscript.

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REVIEW
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Humanized Mice Are Instrumental to the Study of *Plasmodium falciparum* Infection

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Research using humanized mice has advanced our knowledge and understanding of human haematopoiesis, non-adaptive and adaptive immunity, autoimmunity, infectious disease, cancer biology, and regenerative medicine. Challenges posed by the human-malaria parasite *Plasmodium falciparum* include its complex life cycle, the evolution of drug resistance against anti-malarials, poor diagnosis, and a lack of effective vaccines. Advancements in genetically engineered and immunodeficient mouse strains, have allowed for studies of the asexual blood stage, exoerythrocytic stage and the transition from liver-to-blood stage infection, in a single vertebrate host. This review discusses the process of "humanization" of various immunodeficient/transgenic strains and their contribution to translational biomedical research. Our work reviews the strategies employed to overcome the remaining-limitations of the developed human-mouse chimera(s).

Keywords: humanized/chimeric mice, malaria, NSG mice, TK/NOG mice, FRG mice, huRBCs, huHep, clodronate loaded liposomes

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Abbreviations: ACT, Artemisinin-based combination therapy; AIDS, acquired immune deficiency syndrome; anti-Gr1, Anti-granulocyte receptor 1; APCs, antigen presenting cells; cGy, centigray (equals to 0.01 gray); CIMP, Chemical immunomodulation protocol; Clo-lip, Clodronate-loaded liposomes; CYPs, Cytochrom P450s; DCs, Dendritic cells; FAH, fumarylacetoacetate hydrolase; *FoxN1* - forkhead Box N1; FRG, FAH$^{-/-}$Rag2$^{-/-}$IL2Rγ^{null}; GCV, ganciclovir; GM-CSF, Granulocyte-macrophage colony-stimulating factor ; GVHD, graft versus host disease; hBMCs, human bone marrow cells; HepG2, human liver carcinoma cells; hESCs, Human embryonic stem cells; hPBLs, human peripheral blood lymphocytes; HSCs, Human hematopoietic stem cells; HSVtk, herpes simplex virus type 1 thymidine kinase; huBMCs, Human bone marrow cells; huHep, Human hepatocytes; huPBLs, Human peripheral blood lymphocytes; huRBCs, Human-red blood cells; IFA, immunofluorescence assay; IFN-γ, interferon gamma; *IL-2rg*, interleukin-2 Receptor ɤ-chain; IP, Intra-peritoneal; iPSC, Induced pluripotent stem cells; IRS, indoor residual spray; IV, Intravenous; KO, knockout; LS, Liver stage; MCP-1, Monocyte chemoattractant protein-1; M-CSF, Macrophage colony-stimulating factor; MP, Malaria pigments; MST, Median survival time; NICC, Neonatal porcine islet cell clusters; NK-cell, Natural killer cells; NOD, Non-obese diabetic; PBMCs, Peripheral-blood mononuclear cells; PHA, Phyto-haemagglutinin; *Prkdc*^{SCID}, Protein kinase, DNA activated, catalytic polypeptide; Severe Combined Immunodeficiency; qRT-PCR, Qualitative reverse transcriptase polymerase chain reaction; *Rag*1/2, Recombination-activating gene 1/2; RBCs/huRBCs, Red blood cells/human-red blood cells; RH5, Reticulocyte-binding protein homolog 5; rhGH, Recombinant human growth hormone; rhuPRL, Recombinant human prolactin; RI/huHep-RI, Repopulation index/huHep-repopulation index; SCID, Severe combined immunodeficiency; SIRPα, Signal regulatory protein alpha; TB, Tubercuosis; TCR- T-cell receptor; TNF-α, Tumor necrosis factor-alpha; TRAP, Thrombospondin-related anonymous protein; Tregs, Regulatory T cells; uPA, Urokinase plasminogen activator; USGI, ultra-sound guided injection; WHO, World Health Organization.

BACKGROUND: THE BURDEN OF MALARIA

Among the numerous infectious diseases, malaria remains a major health challenge. Malaria is a disease which spreads through the bite of female *anopheles* mosquitoes, who carry the infection moieties (sporozoites) of parasites belonging to the *Plasmodium* genus (1). The greater morbidity and mortality related to human malaria infections reported by World Health Organization (WHO), is due to the wide range of hosts, mainly affecting humans with a high host tropism. 216 million cases of malaria infection was reported across the world in 2016, with a death toll of 445,000. In comparison, 237 million cases were reported in 2010 and 211 million cases were reported in 2015 (2). The death incidence rate in Sub-Saharan Africa is more than 85% in children <5 years of age (3).

The pathophysiology and molecular mechanism of malaria has been revealed, contributing to improving our understanding of malaria biology. The routine culture of an asexual blood stage infection of *P. falciparum* was successfully achieved (4). However, various aspects related to the mechanism of cell motility and invasion, changes in cell signaling pathways and the modulation of host cells, escape from the immune system and establishment of an infection into the liver, or to stay in hypnozoite stages, are not very clear. The technological advances in the field of epidemiology and entomology support the research and reduces the burden of understanding the malaria parasitology by staining the vivid stages of all parasites (5–7).

The tens of millions of non-immune individuals from areas where malaria is not transmitted, visit malaria endemic areas, and face the risk of malaria infection (8, 9). The two major weapons against malaria are vector control and chemoprophylaxis/chemotherapy (10). Various treatments such as indoor residual spray (IRS), use of insecticide-treated bed nets, and medical care through antimalarial drugs (artemisinin-based combination therapy-ACT etc) are available to reduce the burden of malaria (11, 12). Unfortunately, attempts to eradicate the disease based on these methods have had only limited success due to wide spread drug resistance by the parasite (13) as well as insecticide resistance of the mosquito vector (14). This bleak situation drives scientist to develop additional control measures such as a malaria vaccine which is both appealing and urgent. However, an effective malaria vaccine development has not been found, despite enormous and continued efforts made in this direction (15).

Biology of Malaria Parasites

The causative agent of the malaria parasite has a complex multi-stage life cycle which commences with the bite of the female anopheles mosquito, a definitive host that carries sporozoites to infect healthy humans (intermediate host) to complete the life cycle of the parasite (**Figure 1**). The disease in humans is caused by one or a combination of *Plasmodium* spp.: *P. vivax, P. falciparum, P. malariae*, and *P. ovale* (7). Also, in geographically limited zones of South-East Asia, the Malaysian island of Borneo in particular, infections caused by *P. Knowlesi,* a zoonosis without visible transmission to other hosts, can be observed (16).

The systemic *P. falciparum* infection causes greater morbidity and mortality due to its severity and poor diagnosis. On the contrary, *P. vivax* infection is less severe, but formation of hypnozoites and their genesis, need to be studied to understand the mechanism of frequent relapses (17).

Malaria infection in humans begins when the sporozoites from the salivary glands of female *anopheles* mosquito enter the bloodstream via the skin and travel to the liver. The sporozoites traverse through blood vessels and reach the liver, and undergoes the tightly regulated signaling mechanism before rendering the infection to the hepatocytes (1). The invaded hepatocytes allow for the replication of sporozoites and leads to the formation of schizonts. This asexual reproduction stage stays inside the liver of human cells and lasts for 7–10 days, depending on the species and strain of the parasite. Each schizont gives rise to several merozoites which are released into the bloodstream, indicating the end of the exoerythrocytic phase of the malaria infection. The infection by *P. vivax* and *P. ovale* is not characterized with this reproduction step since pathogens might reside as hypnozoites within the hepatocytes for a longer duration, until the infection relapses (18).

The merozoites in the bloodstream invade the erythrocytes and the first stage after invasion is the formation of a ring that later evolves into a trophozoite. The trophozoite cannot metabolize heme so it converts heme into a yellow pigment, hemozoin. The globin part of hemoglobin is utilized as a source of amino acids, needed for reproduction. Trophozoite then develops into erythrocytic schizont and each mature erythrocytic schizont gives rise to new merozoites that eventually rupture the erythrocytes which are released into the bloodstream to invade new RBCs. This is the symptomatic stage in which clinical manifestations of the disease begin to appear. Further, unlike the liver stage, the erythrocytic stage of the malaria parasite repeats multiple times (7, 18).

The differentiation of the parasite into male and female non-pathogenic gametocytes (propagation carriers), also takes place within RBCs. If a female Anopheles mosquito takes a blood meal, the gametocytes are taken up and mature into macrogamete (female) and microgamete (male). The microgamete undergoes three divisions producing eight nuclei in the process in the mosquito's gut, and each nucleus fertilizes with a macrogamete resulting in the formation of an ookinete. The ookinete penetrates the midgut wall and becomes encapsulated, forming an oocyst. The ookinete nucleus divides, producing thousands of sporozoites within the oocyst. Toward the end of sporogony, lasting 8–15 days, the oocyst ruptures and sporozoites travel to the salivary gland of mosquito, ready to be injected into a human host during the next blood meal (7, 19).

HUMAN-CHIMERIC MICE (HUMANIZED MICE)

Mice have been used as *in-vivo* models to study a diverse range of infectious pathogens (20). The murine and human

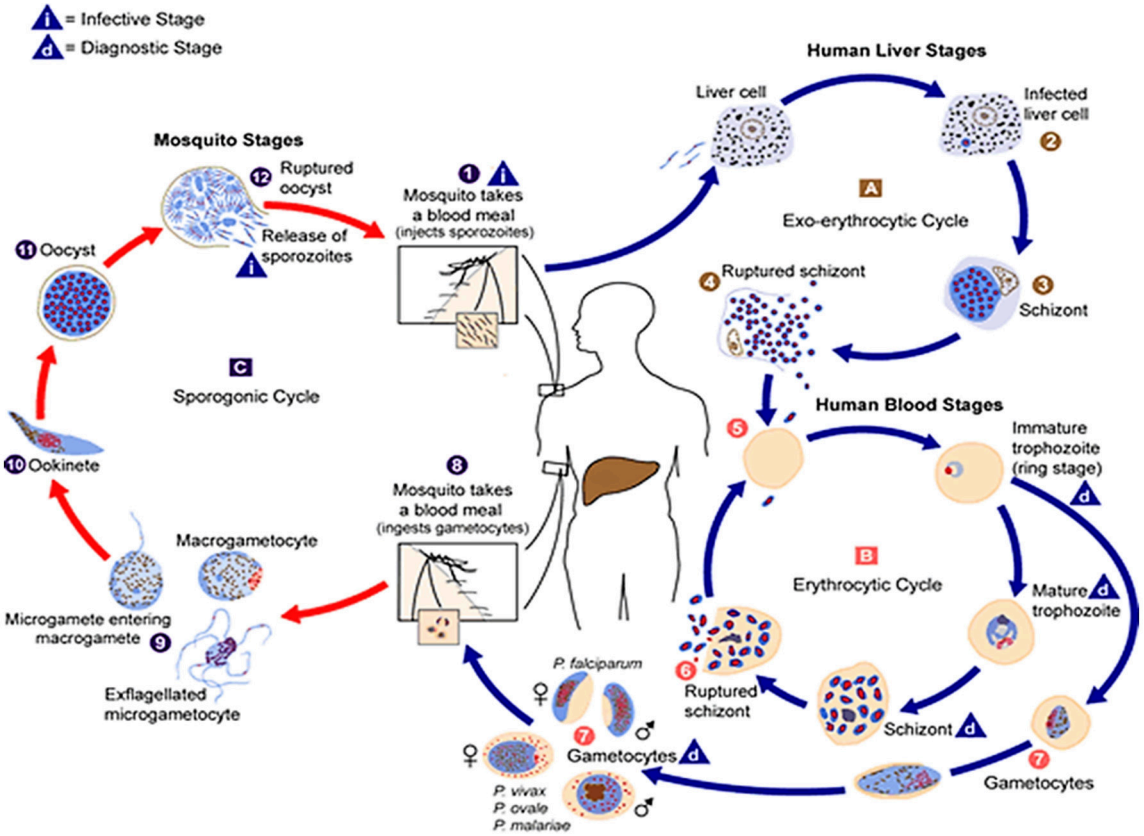

FIGURE 1 | The malaria parasite life cycle 1. Sporozoites infect liver cells 2 and mature into schizonts, which rupture and release merozoites 4. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocyticschizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocyticschizogony B). Merozoites infect red blood cells 5]. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites 5]. Some parasites differentiate into sexual erythrocytic stages (gametocytes) 7. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal 8. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites 12, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites 1 into a new human host perpetuates the malaria life cycle. (Source: https://www.cdc.gov/malaria/about/biology/index.html).

genome shares nearly 85–99% similarity, but due to the complexity of the organization of cells, tissues and organs and specificity of the human immune system (21–23) some mouse strains are less than ideal *in-vivo* models. Recent advances made in translational biomedical research suggest the importance of immunomodulatory agents and target-specificity, and encourage the development of "humanized mouse models" (21, 24).

The theoretical and empirical evidence for the need of "human" mouse models, as well as advancements made in immunological research is the genesis and driving force for the development of chimeric mice. The immunodeficient mice transplanted with human cells or tissues are referred to as "humanized mice," with engraftment and repopulation of a functional human system (25). Since tissue and cell grafts are perceived as foreign and rejected by the host immune system, immunodeficient mice are inevitably required to achieve human cell engraftment followed by their repopulation. Therefore, immunomodulatory agents are administered to modulate the immune response to reduce graft rejection episodes.

Humanized-mice evolved from the origin of athymic (nude) mice with an underdeveloped/impaired thymus. The process of "humanization" of immunodeficient mice has progressed and a variety of immunodeficient mice have been developed by incorporating specific defects in their immune system through genetic engineering approaches. A brief account on immunological and physiological characteristics of some

TABLE 1 | list of various immunodeficient mouse strains.

Mice	Mutation	Mice Information	Character	Application	References
BXN (Beige/Xid/Nude) Developed by the collaborative work of NIH, USA, and referred as "NIH-III"	Beige mutation (bg^J or bg^{2J})	Originally produced at Oak Ridge National Laboratory due to radiation-induced mutation, name represents the color of affected mice Available as a single mutation in C57BL/6J or C3H/HeJ Also available at Jackson Laboratory	Defective and reduced bactericidal activity, absence of NK cells and defects in CTLs and antibody responses to tumor cells Defect in lysosomal enzymes of neutrophils Short life span, low body weight and poor bleeding time	Identical mutant to Chediak-Higashi syndrome in humans which helps in understanding the similar abnormalities To study the role of pigment and their genes in combination with pale ear (*ep* mutation) Study of giant lysosomes an important marker in identification and studying hematopoietic stem cells Most importantly to study the mechanism of metastatic spread of cancer, infectious diseases, hypersensitivity, bone marrow grafts To distinguish between the cytotoxic function of NK cells and NK T cells in normal and beige mice	(32–39)
	Xid mutation	CBA/N mice having X-linked immune deficiency mutation Available at Jackson Laboratory	Depletion in the number of peripheral B-cells and The B cells having less surface IgM to IgD ration which represent the disorder in B-cell maturation Curtail in IgM and IgG3 concentration in the serum, and reduction in capacity (or inactivity) of thymo-independent MHC-II responses	Helpful in understanding the mutation of Bruton's tyrosine kinase gene (*Btk*) which plays an crucial role in B-cell development To study the expression of V_H gene in spleen Reveal the crucial role of IgM in preventing the infection of *Crytococccus neoformans* from lungs to the brain in chronic pulmonary infection model Role of BCR in TACI expression (T cell-independent type 1 Ag such as LPS which stimulate BCR)	(40–44)
	Nude mutation	Mutation in *FOX1* (winged-helix/forkead transcription factor) gene, also called athymic nude Abnormal hair growth First reported at Virus laboratory, Ruchill Hospital, Glasgow by Dr.N.R.Grist Available at Jackson Laboratory	Practical absence of the thymus hairlessness, lack of functional T-cells, and deficiencies in cellular immunity, Partial defect in B cell development However, there is no defect in T-cell precursors and therefore in adult mice some functional T cells can be observed Due to the defect in T helper cell activity, thymus-dependent antigen were detected by IgM only, high NK cell activity	As an experimental model for tumor inoculation to study the metastasis and their growth inhibition by drug treatment Suitable model to study the infectious diseases (*Mycobacterium leprae,* parasite infection of protozoa and worms) Studies of autoimmunity and allergy Model for the liver deficiency study and treatment and xenotransplantation	(40, 45–49)
SCID mice	Mutation in the $Prkdc^{SCID}$ gene on chromosome-16	Mutation in an enzyme which has an activity in DNA repair *Prkdc* (protein kinase, DNA activated, catalytic polypeptide) No V(D)J recombination occur $Prkdc^{SCID}$ naturally occurred in BALB/c-Igh^b which are maintained at Institute of Cancer research in Philadelphia Available at Jackson Laboratory	Non-responsiveness of spleen toward T and B cells making them unavailable Ideal host for xenografting cells originating from the myeloid lineage remained unaffected due to their vital role for the survival which enable to generate lymphocytes bearing receptors of restricted diversity called "**leakiness**" Flawed DNA repair mechanismleave them susceptible toward radiation The first mouse strain that reportedly supported the human peripheral blood mononuclear cells (hu-PBMCs) engraftment to study HIV infection	Useful in studies of normal and abnormal lymphocyte development and function, role of nonlymphoid cells in the absence of lymphocytes Humanized SCID used to study the infectious diseases (HIV and EBV) and their therapeutics Suitable for the engraftment of human tissues such as tumor cells and cell lines, skin, pancreatic islets Ideal model for human tumor xenograft growth, pathophysiology and their metastasis Useful in studying the effect of radiation in normal and tumor cells for genetic deficiency and their repair mechanism	(50–54)

(Continued)

TABLE 1 | Continued

Mice	Mutation	Mice Information	Character	Application	References
RAG mice	carrying a deleted recombination activating gene (*rag1* or *rag-2*)	Mutation in *Rag1* and *Rag2* gene which are important for V(D)J gene rearrangement that can produce the antigen receptor in B and T cells Available at Jackson Laboratory	Inhibits B-cell and T-cell differentiation, results into lack of B and T-cells NK-cell activity is prominent, they do not exhibit immunoglobulin leakiness and less sensitivity to radiation Shorter life span, produce higher amount of B cell lymphomas regularly	Commonly used as models to study the role of the immune system in cancer (tumorigenesis and metastases), autoimmunity, and chemotherapy studies as a potential alternative to Nude and *SCID* mice Higher level of engraftment of human HSC and lymphoid cells Study of the gene function in lymphocyte development by blastocyst complementation method	(55–58)
NOD mice	Contain a unique MHC haplotype (H-2^{g7}), and a single nucleotide polymorphism (SNP) in the TNF-α and CTLA-4 genes	Non-obse Diabetic mice (NOD/ShiLtJ strain)	Impaired immune system with hampered maturation of macrophages, wound healing Defects in their natural killer (NK) cells and $CD4^+CD25^+$ T-cells Deficiencies of NK T-cells and absence of C5a complement	Important model for human type I diabetic study Allow the prolonged engraftment of human cells and tissues used for infectious disease and cancer Useful in the identification of responsible antigen, genes susceptibility for the diseases, effect of environment on the diseases, efficacy of therapeutics, and development of imaging techniques Humanized-NOD mice with higher susceptibility of vivid xenografts make it a suitable model for the translational biomedical research	(59–61)
NOD/SCID mice	Backcross of NOD with SCID mice (such as C3H/HeJ-SCID and C57/BL6-SCID) transferred the $Prkdc^{SCID}$ mutation	Developed by the Fox Chase Cancer center via transferring SCID mutation from C.B-17 to non-obese diabetic background Available at Taconic Bioscience/Jackson Laboratory	Unable to develop diabetes with diminished NK-cell activity and suppressed non-adaptive immune responses Capable of accepting a higher level of engraftment of hu-PBMCs, HSCs, liver and lungs No activity of complements and macrophages exhibiting the defects in the expression of cytokine receptors Shows 80-90% engraftment of HSCs in comparison to other strains Short life span of 8-9 months due to the severe thymic lymphomas, required special care and maintenance, paralysis may occur but changes are rare Leakiness is very less compare to other SCID models and chances of increases are less with the age	Usually used for translational biomedical applications and xenograft transplantation Recommended model for the study of the human platelet survival Study of the pathogenesis of drug induced immune thrombocytopenia (DITP) Useful in cancer biology research for the study of tumor formation, metastasis, their therapeutic treatment and regulation, imaging studies of tumor	(62–70)
NOD/SCID/$\beta_2m^{-/-}$ mice	Knockouts for β2-microglobulin gene which drives the expression and function of murine MHC-I molecules	Double homozygous for SCID and beta2m *B2m* mutation was developed by Dr. Beverly Koller and Dr. Oliver Smithies at University of North Carolina followed by *scid* and *B2m* combined mutation at Jackosn laboratory by Dr. Leonard Shultz	Bear the entire set of deficiencies prominent in the NOD/SCID mice along with complete death of NK-cell activity Disrupts the development and function of NK-cells, B and T-cells, this hampers the MHC-I-mediated immunity Several disadvantages for instance, capacity to sustain only limited development of human lymphocytes, restricted number of T-cells constituted and repopulated in the chimeric organs	Most suitable model for the xenograft transplantation (intra-bone marrow) To study the Influenza infection (due to restricted influenza-specific IgG), myelodysplastic syndrome For the vaccine development and the therapeutics for the tumor	(21, 71–74)

(Continued)

TABLE 1 | Continued

Mice	Mutation	Mice Information	Character	Application	References
NOD/SCID/ IL2Rγ mice	Mutations in the *Il-2rg* gene	Referred as NSG mice NOD/schi-SCID Il-2rg$^{-/-}$ (NSG) mice was developed by crossing the NOD/SCID mice with the *Il-2rg*$^{-/-}$ mice: *scid* and *IL2rg*^{null} (SCID mutation in DNA repair complex mechanism and IL2 rg mutation prevent cytokine signaling via different receptors leading to deficiency in NK cell activity) Available at Jackson Laboratory	Impaired development and function of the lymphocytes and abolish NK-cell formation which in turn impedes IFN-γ secretion Absence of mature murine lymphocytes, functional dendritic cells, leakiness, and prolonged lifespan of mice for about 16 months (long-lived) Various immunodeficient mice were crossed with these IL-2rg$^{-/-}$ knockouts to produce strains with further suppression of immunity	They exhibit excellent ability to accept human xenografts, especially PBMCs, stem cells, myeloid cells and progenitors originated from fetal liver, cord blood, bone marrow and PBMCs Exclusively used for the differentiation and development of HSCs, platelets, RBCs, and T-cells used for analyses Optimum mice model to study the mechanism of inflammation, wound healing, allograft transplantation and their rejection for the clinical purpose (specially human skin graft), remodeling of tissues and revascularization	(75–81)

BCR, B-Cell receptor; TACI, transmembrane activator and calciummodulator and cyclophilin ligand interactor; LPS, lipopolysaccharide; NK cells, Natural killer cells; CTLs, Cytotoxic T lymphocytes; MHC, Major histocompatibility Complex; hu-PBMC, Human peripheral blood mononuclear cells; HSC, Hematopoietic stem cells; RBCs, Red blood cells; SCID mice, Severe combined immunodeficiency mice; Rag1/2, Recombination activation gene 1/2; NOD, Non-obese diabetic.

immunodeficient mice is given in **Table 1** (26). The creation of human-mouse chimera(s) follows a cascade of different steps and events for the reconstitution of human cells, using various immunomodulatory protocols. All the procedures to prepare the host for human cells/tissue engraftment followed by their repopulation were described in detail (27), and have been reviewed many times (**Figure 2**) (21, 28–30) by multiple experts in the field.

The developed mouse-human chimeras present an efficient pre-clinical *in-vivo* model to study the human immune system and its interaction with infectious pathogens, hematopoiesis, stem cell development and function, tumor formation, cancer biology, and regenerative medicine.

Human-Mouse Chimeras Required to Study Infectious Pathogens

The pathogens relevant to human disease, which do not infect other animal species, required an animal model that could reconstitute or harbor human tissues to replicate the human immune system (82). The study of human infectious diseases is restricted to *in-vitro* models as humanization of immunocompromised mice is difficult, due to the scarce data available on host-pathogen interaction, cell behavioral pattern and the biological complexity of the human body. The *in-vitro* effect of drugs and therapeutic effect in animals differ in their metabolic pathways, and does not give an accurate prediction of drug metabolic pathways for humans (83). Drug metabolism in humans and their physiological differences in animal model(s) also make it arduous to design and formulate the efficient drugs and their targets. The development of disease-specific drugs will be possible only when the pathogenicity as well as molecular and immune mechanisms of infectious pathogen is well understood. The testing of preclinical drugs and candidate vaccines require humanized mice that help predict the drug metabolism and pharmacokinetics.

Humanized mice have been used over a long period of time, which helps understand the mechanism of rejection of human tissues provoked by human immune cells. However, translation of these studies from murine (immunodeficient mice) models to the clinic, has limited success due to numerous reasons which demand precious focus on human immune responses to allogeneic tissues along with their cumbersome usages and required skilled personnel (84). The technology for cell and tissue transplantation has improved the reconstitution of human immune cells/tissue that allows for the successful study of graft vs. host diseases (GVHD), different allograft studies and other human immune system studies which have been established as a robust preclinical model for human-specific therapeutic inventions (30, 81, 84). The human immune system may be engrafted into the immunodeficient mice, where type of human cells [pluripotent stem cells and their derived cell populations, regulatory T cells (Treg)] and tissues (human skin, islet, cardiac tissues, xenografts) are important in engraftment (81). Therefore, a robust small laboratory animal model transplanted with human

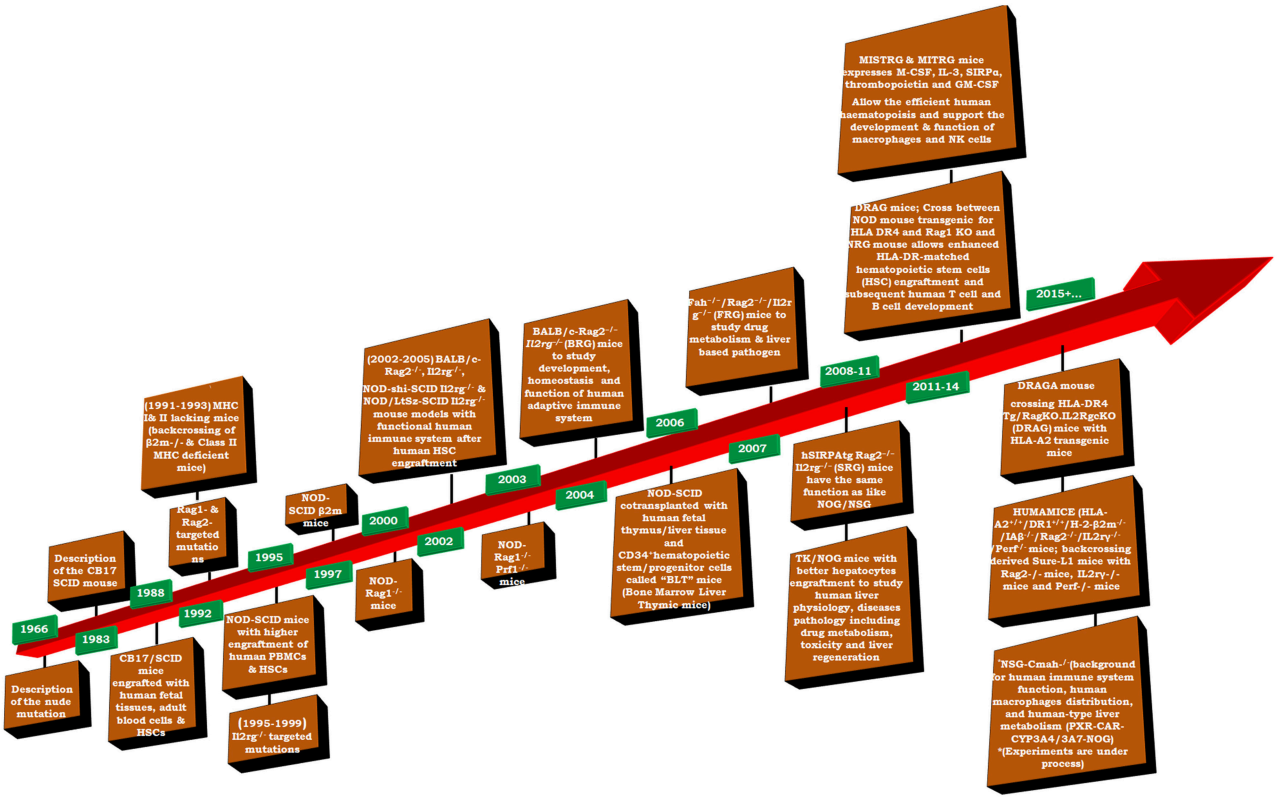

FIGURE 2 | Timeline of important events in the development of humanized mice (adapted and modified from Shultz et al. (31).

hematolymphoid/hematopoietic stem cells is indeed required to advance drug discovery (85).

The liver, an important immunogenic organ, consists of innate immune cells [natural killer (NK) cells, NK-like T cells, Kupffer cells and dendritic cells (DCs)] which play an important role in the local immune surveillance, liver regeneration and pathogenesis of liver diseases (86–88). The enzymes involved in the drug and xenobiotic metabolism and excretion, liver has been the center for the research related to drug mechanism and identifying novel drugs. Hepatotropic viruses and parasitic infections are the cause for a greater rate of mortality in humans, in addition to the toxicity rendered by chemicals. Humanized mice are therefore useful in order to study viral infections with hepatitis B or C and the hepatic stage of the malaria (89) infection. Acute liver failure becomes a predominant issue because of the drug-induced liver injury (90) and uncharacterized behavior of the drug metabolic pathway and clearance from the human body. Therefore, human liver reconstituted immunodeficient mice have been deployed to study the drug metabolic pathways (90, 91). Several approaches have been deployed, including the *in-vitro* culture of human cell lines, transgenic insertions of human genes, cultures of human hepatocytes and chimeric mice with the repopulated humane liver. The *in-vitro* culture has the limitation of continuity which cannot delineate the human liver metabolism. Despite several advantages, expression of transgene, inhibitor and activator proteins of the cells and cofactors required for the reactions derived from the mouse, are some of the limitations (89). To overcome these limitations, investigators have employed human hepatocytes in the culture to investigate the human-relevant drug metabolism processes (89, 92–95). Earlier studies confirmed that hepatocytes are important for the immune tolerogenic properties of the liver (96–98) and their transplantation inhibits allograft rejection (99). Although results obtained by the human hepatocytes with respect to the drug metabolism, studies were limited to the short duration, a maximum of 30–40 days (100, 101). Recently, transgenic mice (TK/NOG) conditioned with ganciclovir (GCV) treatment have shown to deplete a number of mouse hepatocytes to create receptive stroma for the injectable human hepatocytes (huHep). huHep reconstituted humanized mice have been used to test the drugs to predict their pharmacological and toxic nature on human liver (83, 102). It was later observed that human chemokine production by the engrafted huHep is one of the mechanisms that improve the human immune systems reconstitution in the liver of LTH hu-mice (human FTHY and $CD34^+$ HSPCs) (103) since they do not, or respond poorly to mouse counterparts (104, 105). These results indicate the improved human immune system reconstitution in the liver of LTH hu-mice (103).

Humanized mice are used to study different diseases, which have been reviewed several times (20, 25, 29–31, 74, 106–109) along with necessary modifications to overcome the limitation of respective models (24, 70, 81).Therefore we did not give a detailed account on mouse models developed to study other diseases, and focused only on the humanized mouse model(s) used to study the biology of *P.falciparum*.

HUMANIZED MICE ARE INSTRUMENTAL TO STUDY THE *P. falciparum*

Advancements made in biomedical research are not sufficient to fight systemic inflammatory diseases such as *P. falciparum* malaria infection in humans. The mouse-human chimeras have been used to study the asexual blood stage, exo-eryhrocytic stages (liver stage), and transition from liver-to-blood stage (17, 110–113) infection of *P. falciparum*. However, specific immune-responses against antigens produced in all stages of the parasite are yet to be explored (114, 115). And, the stage specificity of human parasites does not allow them to grow and develop in murine models (116).

Rodent parasites (*P. berghei* and *P. yoelii*) are used as surrogates to study the pre-erythrocytic infection of human malaria parasites. The species mismatch between the rodent and human parasites makes it difficult to extrapolate (116) their data for human studies. However, there are syntenic proteins such as hepatocytes surface protein CD81 which helps to render the infection in a rodent parasite and *P. falciparum* (117). Similarly, a sporozoites surface protein named thrombospondin-related anonymous protein TRAP is required for liver infection in the human malaria parasite (118) and rodent parasite (*P. berghei*) (119). On the other hand, hepatocytes growth factor receptor (C-Met) plays an important role in the early pre-erythrocytic infection of the liver by *P. berghei* (120). This receptor is not important to study the biology of *P. yoeli* or *P. falciparum* (121). Likewise, a merozoite adhesive erythrocytic protein MAEBL is required for the liver stage infection in *P. falciparum* by sporozoites (122) whereas it is not required in case of *P. berghei* (123). The human malaria parasites were never seen to infect the murine hepatocytes (112). Therefore, a humanized mouse is required to model *P. falciparum* to understand its basic biology, pathophysiology, immunology and pharmacology.

The pre-erythrocytic stage of the parasite life cycle is asymptomatic, and all clinical pathological symptoms are shown because of the progression of the asexual blood stage infection. The erythrocytic stage is routinely studied *in-vitro* by the development of a continuous culture system that allows the replication and growth of an asexual blood stage parasite within human RBCs (4, 124). Recent advancements in the *in-vitro* culture of *P. falciparum* has expanded the knowledge of its blood stage to show the basic parasitology (5) and the development of target specific drugs effective against asexual blood stage infection (125).

The sporogonic stages are generated by feeding the female *Anopheles* mosquitoes on *in-vitro* gametocyte cultures, and the progression of the parasite life cycle in the mosquito as well as subsequent sporozoite accumulation in the salivary gland of the mosquito is allowed. However, the *P. falciparum* liver stage (LS) has proven much more difficult to study even though complete LS development in primary huHep and subsequent transition to *in-vitro* erythrocytic infection was shown over 30 years ago (126) followed by subsequent studies (118, 127–129). The primary human hepatocytes, or HepG2 cells, have been used to study the development of LS infection of *P. vivax* (130, 131). FRG-KO huHep mice were used to study the LS infection and formation of hypnozoites during the *P.vivax* infection (132).

Currently, drug resistance against the *Plasmodium* strains remains the main hurdle in effective vaccine development. However, there is remarkable improvement in the development of novel drug therapies. On the other hand, development of novel tools are a prerequisite of the rapid transition of preclinical drugs to the clinic, along with the fulfillment of SERCaP (Single Exposure Radical Cure and Prophylaxis) guidelines (133). To address the above-mentioned issues, recently developed humanized mice helped investigating the different stages of malaria parasites in detail (6).

Humanized Mice Support the Development and Replication of Asexual Blood Stage Infection of *P. falciparum* (RBC Reconstituted Humanized Mice)

The human-red blood cells (huRBCs) host the asexual blood stage infection of *P. falciparum*. The huRBCs reconstituted immunodeficient NSG mice present nearly an ideal model to develop an understanding of the asexual blood stage infection. The different strains of immunodeficient mice, which were used for the asexual blood stage infection, are listed in **Table 2**. Different strategies provided a humanized environment for the circulation of human immune effecters and huRBCs, maintaining the stable long-term blood chimerism (17). The sizeable and long-term huRBCs grafting (human blood chimerization) and control of inflammatory responses provoked by the parasites are crucial factors contributing to the success of humanized mouse (134). The same humanized mouse (PfhuRBC-NSG-IV) was used to experimentally induce the artesunate resistance and to validate the resistance phenotype (135).

Other Blood Stage Humanized Mouse Models

Tsuji et al. first reported the transplantation of huRBCs into immunodeficient SCID mice (140). To overcome the rapid elimination of huRBCs from the mouse's periphery, they discovered that the intraperitoneal (IP) injection of human serum significantly extended the survival of huRBCs injected intravenously (IV). After the successful and stable engraftment of huRBCs into SCID mice, some of the animals were irradiated (dose of 300 cGray) 2 days prior to *P. falciparum* infection (FUV strain) and a blood smear was drawn for the Giemsa staining. They observed the rapid elimination of infected and non-infected huRBCs from the non-irradiated SCID mice, compared to the irradiated mice with a decrease in the parasitemia during the initial 5 days of infection. The trend of parasitemia remained constant even upon dilution, by administering huRBCs up to 20

TABLE 2 | The developed "humanized mouse model(s)" for asexual blood stage infection of *P. falciparum*.

P. falciparum strain(s)	Humanized mice	Applications	References
NF54 or multiresistant T24	BXN (Beige/Xid/Nude)	First rodent model for *P.falciparum* study, to test the novel compounds for the drug development and evaluation of drug responses, vaccine development	(136–138)
FUP, NF54, 3D7, Dd2 and clinical isolates	SCID (Severe Combined Immunodeficiency) and NOD/SCID	Helpful in *in-vivo* studies of human malaria parasites and vaccine development	(139–141)
Mouse-adapted 3D7	NOD/SCID/$\beta_2$microglobulin $(\beta_2m)^{-/-}$	*in-vivo* experimental drug/exposure-response assay	(142)
Mouse-adapted 3D7	NOD/SCID/IL-2 receptor γ chain $(IL2R\gamma)^{null}$or NSG mice	To check the response against various antimalarial therapeutics	(143, 144)
Different parasite strains (3D7,UPA and K1) without any prior adaptation	NOD/SCID/IL-2 receptor γ chain $(IL2R\gamma)^{null}$ or NSG mice with an additional treatment of clodronate delivered through liposomes	Most comprehensive humanized mouse model allowing to develop the sexual stage parasites besides the asexual-blood stage development	(134, 145)
NF54, 3D7	HLA-transgenic mice	Discovery of novel protective malaria antigen and immune responses	(146)
NF54	TK/NOG	Used to assess the drug toxicology and metabolism, and *P. falciparum* infection in transplanted human hepatocytes	(6)

TK/NOG, thymidine kinase/NOD/Shi-scid/IL-2Rynull mice; HLA, human leukocytes antigen; 3D7, NF54, PAM, different P .falciparum strain.

days. This report suggested that *P. falciparum* could grow and develop only in huRBCs (140). However, the SCID mice needed further modifications to establish the hosting of huRBCs. This mouse proved to be a valuable tool for the *in-vivo* study as well as the vaccine development of human malaria parasites.

Similarly, Moore *et al* used the NOD/SCID mice infected with NF54 and 3D7 *P. falciparum* strains, with a single dose of uninfected huRBCs, injected intraperitoneally to see their development in the mouse's circulation. Later, infected and un-infected huRBCs were injected intrapertioneally every day. They observed the stable human blood chimerization and parasitemia in the peripheral blood for 16 days post-infection. Also, the sexual form of parasites (gametocytes) were observed and assessed with respect to their infectivity (139). In conclusion, NOD/SCID mice could also serve as a model for humanization without using any immunomodulatory agent. This might help in understanding the role of the immune response against the *P. falciparum* infection, but the adaptation of parasites and ascetic solutions prior to IP injection was required.

Later, Badell *et al* suggested the use of AB^+ huRBCs to achieve a significant blood chimerism in SCID/NIH III mice for extended periods. However, they used different immunomodulatory agents to deplete the host's residual innate immune effecters. The dichloromethyleneblphosphonate-(CI_2MBP)-encapsulated liposomes were administered weekly intraperitoneally. The parasitemia was estimated up to 3% and maintained over 0.3% for 17 days. All the developmental stages of asexual erythrocytic cycle were observed. Therefore, this mouse model was used to study the drug(s) at pre-clinical levels for the development of a vaccine (136) and to assess the effect of antimalarial drugs reported for human infection (138). These findings overcame the limitations seen with previous models deploying novel immunomodulation protocols.

To develop an ideal mouse model to carry out wider applications of *P. falciparum,* Sabeter *et al* used CIMP (chemical immunomodulation protocol) in NOD/SCID mice to see whether they are susceptible and receptive to infection. They compared their model with the existing huRBCs-NIH III (BXN) mouse. The CIMP was modified and infected huRBCs, with various stages of parasites injected into the NOD/SCID mice intraperitoneally and followed until the end of the study. PCR and Giemsa staining were used to determine the low-grade parasitaemia together with a histopathological analysis. The infected percentage of NOD/SCID and BXN was estimated at 75 and 8%, respectively for 35 days post-infection. The higher-grade parasitemia in BXN and initial lower parasitemia was the hall mark in NOD/SCID for 7 days. The greater rise in the parasitaemia was seen during the second infectious challenge on day 17. PCR was performed on the DNA extracted from the animals, which showed 100% sensitivity and 67.3% specificity. The histology on different organs such as the bone marrow, spleen, liver, lungs and kidney was carried out. The malaria pigments (MP) inside the phagocytic cells were responsible for the elimination of infected-huRBCs, from the peripheral blood used as an indicator. These results suggested the distribution of MP laden macrophages were dependent on the parasitaemia and mouse strain (149). In brief, treatment of CIMP showed the better engraftment of huRBCs in NOD/SCID mice and supported the development and progression of *P. falciparum* infection. NOD/SCID mice were used to test the anti-malarial activity of novel compounds. The susceptibility of NOD/SCID mice with two different *P. falciparum* strains (3D7 and Dd2) and clinical isolates were assessed using slightly modified immunomodulation protocols. Results showed the parasitaemia of 0.05–8% sustained for 19 days with the development of asexual and sexual stage of parasites. These findings suggest that prior adaptation of parasite to its host is not necessarily required for the growth and development of the parasite (141).

Recent Development and Currently Available Humanized Mice for Asexual Blood Stage

The usage of chemical agents may influence the parasites development as well as the unexplored interaction with antimalarial drugs. Thus, without the use of any additional

immunosuppressive agents, the successfully developed NOD/SCID/$\beta_2$microglobulin $(\beta_2m)^{-/-}$ mice paved the way for the asexual stage of *P. falciparum* to examine the antimalarial compounds and the human response (142).

The mouse-adapted *P. falciparum* strain was used to study the infectious challenge in a novel mouse model "NSG mice" (NOD/SCID/IL-2 receptor γ chain $(IL2R\gamma)^{null}$). These mice supported the ten-fold higher burden of parasitized and non-parasitized huRBCs, compared to the NOD/SCID/$\beta_2$microglobulin $(\beta_2m)^{-/-}$mice (144). Although administration of huRBCs intraperitoneally presents better engraftment (142, 143), the transperitoneal passage of blood from the peritoneum to the blood stream was one of the major issues observed with the IP model (134). The value of this mouse model was validated by the assessment of the therapeutic potential of antimalarial drugs (147).

The optimal blood stage mouse model was developed by employing different malaria parasite strains, without requiring prior adaptation to the host. The huRBCs injection and infectious challenge were administered through intraperitoneally and intravenously (134, 145), respectively. The high rising and long-standing parasitaemia was achieved in the mouse by controlling the number of monocytes/macrophages through the treatment of clo-lip (**Supplementary Figure 1**). This pharmacological agent was shown (145) to deplete 70–80% murine monocytes/macrophages. The model also demonstrated a higher level of parasite synchronization and partial sequestration of tainted huRBCs in the vasculature, a hallmark marvel observed in malaria patients. The huRBCs reconstituted mouse showing sustained and stable development of asexual blood stage parasites, also supports the development of sexual stages (gametocytes) (**Supplementary Figure 2**) (145). The developed humanized mice was used to induce the experimental induction of high level artesunate resistance in *P. falciparum* and to validate the resistance phenotype (135).

The huRBCs reconstituted NSG mice were challenged with *P. falciparum,* showing the development of gametocytes. Further, the developed mouse model allowed the *in-vivo* experimental drug/exposure-response assay (147). Developed humanized mice, harboring asexual blood stages of *P. falciparum,* more closely resembled the process in humans. Later, huRBCs-NSG mice were used to understand the interaction of gametocytes with the bone marrow and spleen and the efficacy of anti-gametocytidal activity of drug(s). The value of this mouse model was validated by assessing the efficacy of primaquine, which kills the sexual stages of *P. falciparum.* The clearance of gametocytes, compared to that seen in the control, suggests that the immunomodulation protocol does not hinder the assessment activity of drug effectiveness against the transmission stage of the *P. falciparum* infection. This helps better understand the role of drugs on the gametocytes present in the sequestration sites (150).

The supply of human serum and hypoxanthine resulted in the augmentation of a greater huRBCs chimeric index and parasitic growth. The continuous supply of huRBCs with an effective immunomodulatory protocol is inevitable for the effective and successful growth and development of the *P.falciparum* infection in available humanized mice. The issue of poor huRBCs grafting was overcome by the administration of human cytokines (151). The gamma irradiated NSG mice were injected with $CD34^+$ HSC intracardially and showed more than 40% human leukocytes reconstitution administered with the plasmid containing erythropoietin and human IL-3 intravenously (152). Mice showing at least 1.5% huRBCs reconstitutions were used for the subsequent experiments. Purified mature schizonts (3D7 strain) of the *P. falciparum* (*ex-vivo* infection) were added to the blood, maintained in RPMI 1640, supplemented with serum and incubated at 37°C for up-to 64 hrs, followed by the Giemsa staining. Within 16 hrs of infection, ring stages were observed with the parasitaemia of 0.02 to 1.6%. The infected-RBCs harvested from the humanized mice were analyzed by fluorescent activated cell sorting. The infectious challenge in *de-novo* synthesized and reconstituted humanized mice were detected by microscopy, PCR and flow cytometry. This shows the variation in the infection ability of various strains tested. The *P. falciparum* infection in the *de -novo* generated huRBCs as well as re-invasion was observed (151).

Sexual Stages and Blood-to-Mosquito Transmission

To determine the infectivity of the developed sexual parasites in huRBC-reconstituted NOD/SCID mice, *Anopheles stephensi* and *A. freeborni* mosquitoes directly fed on mice showing sexual stages. The oocyst formation in the mid-gut of the mosquitoes 7–10 days post-feeding, indicates the infectivity of sexual parasites and their successful transmission from humanized mice to mosquitoes (139). Similarly, the FRG-NOD huHep mice successfully produced the asexual blood stage parasites when they were challenged with NF54 *P. falciparum* infection. Subsequently, parasites were maintained in a continuous culture and produced gametocytes. The mosquitoes fed on these sexual stages, and upon dissection, the number of oocyst and sporozoites were estimated as the same in the salivary glands of infected mosquitoes (111). Later, the sexual parasites developed in other humanized DRAG mice were investigated for the transmission to mosquitoes via direct infection or through an *in-vitro* culture method. The development of oocyst and sporozoites in *A. stephensi* mosquitoes in both conditions was observed (146). Further, TK/NOG mice showed the development of all stages of gametocytes and maintained it for a longer duration. However, their transmission to mosquitoes required further experimental validation (6).

Liver-Humanized Mice to Study Exoerythrocytic (EE)/Liver Stage Infection of *P. falciparum*

From the multistage life cycle of the human malaria parasite in their primary (mosquitoes) and secondary host (human), the least known stage is the exoerythrocytic (EE)/liver stage. Notably, the exploration of the LS infection of *P. falciparum* in a human host is hampered by the low success rate of a hepatic stage culture, which is limited to a few days and which can be extended up to a month (128). This is possible with *P. falciparum,* by *in-vitro* human hepatocytes alone or through the co-culture (129, 153) in HC-04 cells, but with very low sporozoites infectivity estimated at 0.066% with *P. falciparum,* posing a challenge to

study LS infection (127). The infectivity of sporozoites were later seen to increase up-to 0.8% (122, 154) and 1.3–1.4% in the same hepatocytes cells (HC-04) during the study of protein O-fucosylation activity (118), which is still relatively low compared to what could be achieved with primary hepatocytes or with *P. berghei/yoelii* infection in *in-vitro*. Therefore, the development of the more convenient *in-vivo* model will help better understand the liver pathophysiology within a human host. A transgenic and immunodeficient mouse strain showing better control over its non-adaptive immune response is therefore needed. The chimeric mouse model(s), as reported elsewhere (109), facilitates the understanding of LS and mimics humans to develop a further understanding of the various biological phenomena of LS parasites.

Liver Stage Mouse Models: Developments and Recent Advances

Following this direction, the first attempt was made to transplant the huHep in SCID mice (110). huHep were transplanted through open mouse survival surgery, followed by an infectious challenge of infected sporozoites isolated from the *A. stephensi* mosquitoes. The histopathological studies were carried out on sections of various tissues removed on day 3, 7, and 9 post-infectious challenge. Immunofluorescence assays were carried out with circumsporozoite protein (CSP), merozoite surface antigen 1 (MSA-1) and liver stage antigen-1 (LSA-1) to detect the *P. falciparum* infection in transplanted hepatocytes. The success rate of transplanted huHep-SCID mice were estimated at around 95% and infection was detected even 4 months after human hepatocyte transplantation. The results obtained were similar to that of non-human primates such as chimpanzees. However, the rate of sporozoites infection and their correlation with other existing animal models need to be evaluated (110).

Liver with severe chronic disease conditions, can enhance the engraftment of huHep (155). The expression of uPA was back-crossed with SCID or SCID/Beige background which resulted in the better engraftment and repopulation of huHep, measured up to 14 weeks at regular interval post-transplantation. The proteomic analysis was performed to evaluate whether the huHep were functioning properly through COFRADIC^{TM}. uPA/SCID-huHep mice were given the infectious challenge with the *P. falciparum* sporozoites, and the liver of infected animals was extracted for immunofluorescence assay to carry out a gene expression study. Several specific antibodies, reported for parasites, were used to detect the *P. falciparum* infection. Furthermore, RNA was extracted from the liver tissues to see the expression of specific genes such as PfCSP, PfLSA-1, and PfMSP-1, present during the early and late liver stage infection (156). The longer period of survival of parasites within the transplanted huHep were questioned. Therefore, residual innate immune effecters were controlled by the IP injection of clodronate-liposome. Immunohistochemical studies have shown higher engraftment of human hepatocytes within the clusters of hepatocytes. The intravenously injected sporozoites were seen to infect huHep as well as the development of schizonts on day 5 in the sections made from liver tissue and stained with the Hematoxylin-Eosin stain. The use of different antibodies and gene expression studies confirmed the development of liver stage infection (157). The role of sporozoites-expressed genes P52 and P36 (important for the hepatocyte infection) in genetically attenuated parasites (GAPs) showed the development (158) and expression of liver stage antigen-1 (LSA-1) during the *P. falciparum* infection (159) in uPA/SCID humanized mice. Also, uPA/SCID humanized mice were used to better understand the migration of sporozoites inside the human host and to study the escape mechanism of SPECT (sporozoites microneme protein essential for cell traversal) and PLP1 (perforin-like protein 1) employed by *P. falciparum* sporozoites (154). However, the poor breeding efficiency, a limited time-window for transplantation, renal diseases in reconstituted mice (160) and liver injury caused by the plasminogen treatment which leads to the other complications, were major concerns (148). Additionally, mice were not used to study the transition of parasite from liver-to-blood stage infection in one host (147).

Grompe et al developed a model with a mutation in the fumarylacetoacetate hydrolase (FAH) enzyme. These mice however, died within 12 hrs of birth, due to liver complications (161). Therefore, these FAH-KO needed continuous drug (NTBC) pressure to avoid liver damage and associated health complications (162). Thereafter, several mouse models were developed by back-crossing FAH knockout mice with NOD/SCID or RAG1. None were able to repopulate the human hepatocytes. Besides, FAH/nude, and FAH/RAG1 were also unable to engraft the human cells due to the immune rejection (160). Nevertheless, FAH/NOD/SCID mice were grafted with a smaller number of human cells. However, liver failure resulted in the death of animals when NTBC pressure was withdrawn (160).

Liver Stage Mouse Model and Transition From Liver-to-Blood Stage Infection

As Rag2$^{-/-}$*Il2rg*$^{-/-}$ mice have shown the highest engraftment, FAH$^{-/-}$ were backcrossed with Rag 2 and IL2Rγ and resulted in the triple knock-out (KO) of FRG mice (FAH$^{-/-}$Rag2$^{-/-}$IL2Rγ^{null}). This mouse model achieved more than 90% engraftment of huHep and several mice survived up to 4 months, even after the withdrawal of the NTBC drug, which was confirmed by histological, immunocytochmeical and RT-PCR analysis specific to human hepatocytes (160). The FRG-huHep chimeric mice were injected with *P. falciparum* sporozoites intravenously. Mice were euthanized on day 3, 5, 6, and 7 post-infection for histopathology, indirect immunofluorescence assays (IFAs) and gene expression studies. Results of all the studies confirmed the successful infection in transplanted huHep, transcripts of *P. falciparum,* which amplified specific genes, and the development of LS infection (111). The formation of mature merozoites followed by the transition to the blood-stage is a very important step, as it allows for the study of a complete life cycle in one host. C57BL/6 background of the FRG mice hindered the engraftment of huRBCs because of incompatibility with SIRPα (163). Therefore, FRG, and NOD/SCID mice were back-crossed to create "FRG-NOD" which was reconstituted with huHep and challenged with *P. falciparum* sporozoites followed by intravenous injection of

huRBCs on the 6th and 7th day post LS infectious challenge. The huHep reconstituted mice showed the development of LS infection (111). Subsequently, blood was processed with an *in-vitro P. falciparum* culture medium to cultivate the parasites. The blood smears were drawn from the culture and stained with Giemsa solution every 24 h. The gametocytes along with different asexual stages of parasites were observed. This indicates the successful transition from liver-to-blood-stage infection (111).

FRG-huHep mice were used to assess the humoral immune response against the LS infection of *P. falciparum* (164). The natural infection route (mosquito's bites) was chosen, rather than adopting the conventional route of administration of sporozoites. FRG-huHep mice have revealed the role of inhibitory antibodies against the *P. falciparum* infection. This humanized mouse model could be used to optimize and assess the efficacy of candidate vaccines (164) prior to the clinical trials. Recently, FRG-huHep mice were used to determine the anti-liver stage potential of Atovaquone-Proguanil and Primaquine (165). A novel antimalarial molecule (DSM265), which reportedly selectively targets *Plasmodial* dihydroorotate dehydrogenease (DHODH) *in-vitro* and *in-vivo* blood stage and improve liver stage activity in *in-vitro,* was tested in these mice (166). The oral dose of the drug quantified the log reduction to 2.5 in the liver parasites by *in-vivo* bioluminescent imaging through an IVIS spectrum. The obtained results helped to understand the mechanism of action, the pharmacokinetic or pharmacodynamic study of drugs, before deploying to clinical trials (165).

Likewise, FRG-huHep mice with slight modifications in the administration of huRBCs were used, with the additional treatment of the cytotoxic chemotherapy agent cyclophosphamide with clo-lip, to prevent the phagocytosis of huRBCs (167). This revealed the effective long terms survival of transplanted mice, allowed the infection in huHep, as well as the higher number of infected huRBCs on day 7 post-sporozoite infection challenge, a successful liver to blood stage transition and a sustained asexual blood stage infection of *P. falciparum* with increasing parasitemia. The *P. falciparum* reticulocyte-binding protein homolog 5 (RH5) is an important merozoite invasion ligand which interacts with huRBCs and is essential for the infection of all the parasite strains (168). Therefore, the anti-RH5 antibody in FRG-huHep/huRBC mice were used to determine its efficacy and utility. qRT-PCR, IVIS and parasite multiplication rates (PMRs) were observed to determine the potential of blocking the blood stage infection *in-vivo* while transitioning from the liver stage (167). The FRG mice, despite allowing the successful development of liver and blood stages, and despite being helpful in the assessment of the efficacy of various novel candidate vaccines and drugs, need skilled personnel to maintain the higher level of huRBCs on day 6 post-LS challenge (167).

Next Generation Mouse Models for Exoerythrocytic Stage and Completion of Life Cycle in One Host

To overcome the limitations of uPA/SCID and FRG mice, a transgene called herpes simplex virus type 1 thymidine kinase (HSVtk) was expressed in the hepatocytes of NOG mice (TK/NOG). The host was prepared by GCV conditioning which induces the apoptosis of liver cells to deplete the number of murine hepatocytes. The reconstitution of GCV conditioned TK/NOG mice show the sizeable huHep-repopulation index (huHep-RI) confirmed by the immunohistochemical staining of different antibodies. The value of developing human liver chimeric (huHep-TK/NOG) mice was assessed by determining the efficacy of human specific drug metabolism in these mice. The long-term survival (more than 6 months) without any exogenous drug treatment justifies the value of huHep-TK/NOG to study the LS infection of *P. falciparum*. TK/NOG mice allow the additional dose of GCV even after the human cell repopulates, which helps achieving higher re-population index of human xenografts (148). Additionally, TK/NOG mice hardly require additional drug supplements to suppress the host immune system, except the residual cells of the monocyte-macrophage lineage (169). huHep reconstituted TK/NOG mice favor and support the development of LS and transition to blood stage infection.

Studies were carried out with the huHep-TK/NOG mice to gauge the metabolism of anti-inflammatory drug (diclofenac) in humans (170). These mice revealed the potential for reliable 2-fold engraftment, for all phases of the *P. falciparum* life cycle within one host. huHep-TK/NOG mice supported the huRBCs reconstitution at days 5–6 post sporozoite challenge in the same host, which allowed for the transition from LS–to blood-stage infection of *P. falciparum* (**Figure 3**) to be studied. The added advantage with this mouse model, unlike others (111), is that it does not need two mouse strains to get the complete life cycle in one host.

Soulard V *et al.* and his group used the TK/NOG mice to study the LS infection of *P. falciparum* (6). huHep-TK/NOG mice were injected with sporozoites intravenously and liver sections extracted from the infected mice were analyzed on the 5th and 7th day post-infection. The parasite development was confirmed specifically in the huHep region. huHep reconstituted mice receiving LS infection when administered with huRBCs intraperitoneally daily, reached 50–60% blood chimerism within the 6th day and 80–90% on day 12. On the 6th day post-LS infection, the transition from the liver to blood stage in the same host was observed. The blood sample was collected and maintained in *in-vitro* culture for 8–10 days to reveal the *P. falciparum* blood stage parasites. The aggregate gametocyte numbers could constitute up to 8.5% of total parasites, and 2.5% mature gametocytes (6). It was revealed that the TK/NOG model, most closely resembles human processes and is therefore the most suitable for preclinical drug studies and *in-vivo* LS infection.

Various humanized mouse models used to study liver-stage infection is summarized in **Table 3** (147).

Human-Immune System Repopulated Mouse Models to Study the Liver Stage Infection

Humanized mice with a human immune system (HIS) will help to develop pre-clinical models for the study of infectious diseases. Mice with the HIS expression called DRAG (HLA-DR4.RagKO.IL2RγcKO.NOD) were employed to study the complete life cycle of *P. falciparum* when transplanted with $CD34^+$ HSCs (146). After 4 months post-transplantation with human HSC, DRAG mice were challenged with *P. falciparum*

sporozoites. The parasitaemia was measured by PCR as well as counted on the Giemsa stained blood smears. The liver sections were observed 5 days later by using immunohistochemistry for HSP70. The blood stage parasites were observed between 10 and 28 days post infectious challenge, followed by the blood stage *in-vitro* culture wherein rings, trophozoites and schizonts were observed (146). The complete life cycle of *P. falciparum* in one host was achieved, but low parasitaemia levels, the development of a functional human immune system and host monocytes (in DRAG mice) and adaptation of the parasite strain(s), are concerns which need to be addressed (6). The recently developed humanized DRAGA mice were injected with the liver *P. falciparum* sporozoites (Pfspz) along with Chloroquine diphosphate pressure (171) to elicit the pre-erythrocytic immunity. These animals were protected against the challenge with infectious Pfspz, but had no protection against the asexual blood stage infection (171). We believe this humice could serve as a pre-clinical tool to study the immunogenic potential of newly discovered malaria candidate vaccines and drugs.

Very recently, NSG mice were transplanted with primed human spleen cells (hu-Spl-NSG) to assess the immune response and to understand the biology of the pathogen, to develop an effective vaccine (172). The two different constructions of the liver stage antigen-3 (LSA-3) (shorter and longer) which express during the liver stage and on the surface of sporozoites used as anti-LSA-3, may prevent the invasion of hepatocytes by sporozoites and protect them. Both constrains of protein have shown the different results, as the shorter form of LSA-3 shows the production of IFN-γ (humoral immune response) which was null in the full length protein, whereas the full length of LSA-3 protein showed the production of T regulatory (Treg) cells, but not a shorter constrain. The similarity of Treg sequences found in the human samples was confirmed. hu-Spl-NSG mice might be used to estimate the immune response, and overcome the limitations of graft vs. host reactions seen in hu-PBMCs reconstituted mice (172).

The complete cycle of (LS to blood stage transition) *P. falciparum* in developed humanized mice, will help performing numerous applications for vaccine development and formal genetic studies. In conclusion, developed human liver chimeric mice will revolutionize translational biomedical research and open doors for the vaccine development and drug therapeutics for other inflammatory diseases such as hepatitis, dengue, cancer, AIDS, and TB.

CONCLUSION AND FUTURE PERSPECTIVES

Considering the importance of humanized mice in biomedical research, coordinated efforts are being made to engineer the advanced immunocompromised mice, to support human cell (HSCs and hepatocytes) grafting. The introduction of genetic immunodeficiency in mice plays a central role during "humanization" to reduce graft rejection episodes. Therefore, various mouse strains have been engineered to deplete the host's immunity, which aims to achieve sizeable grafting of

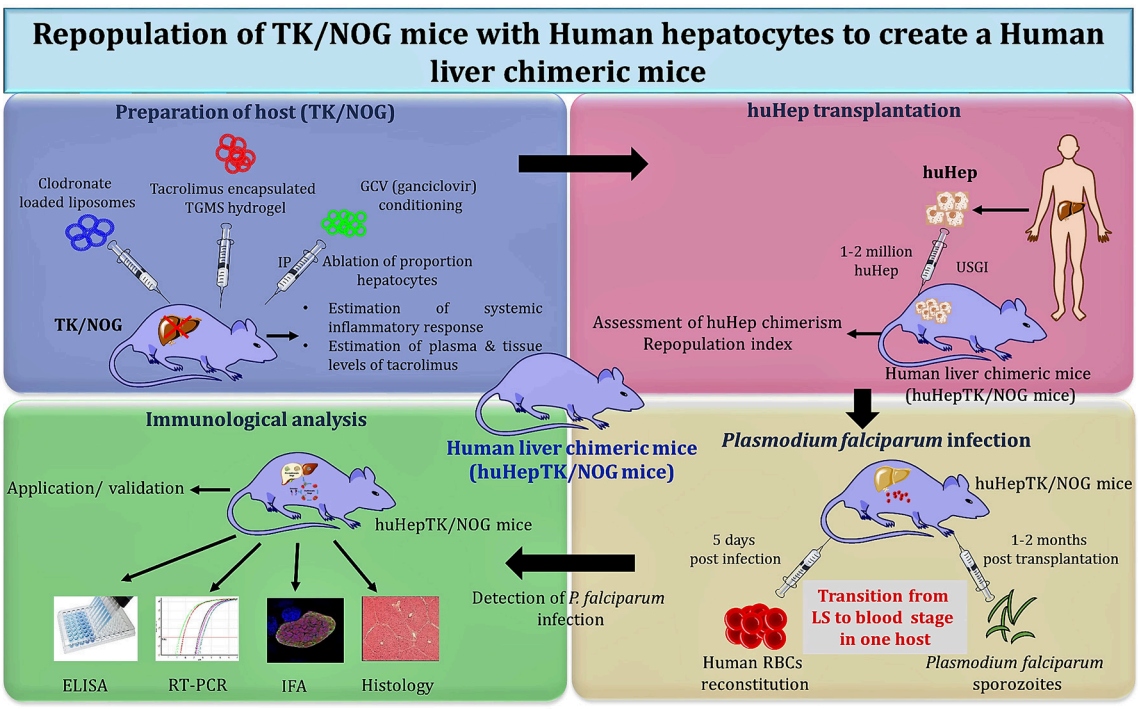

FIGURE 3 | Schema of "creation" for human liver chimeric mice to study LS infection of *P. falciparum* and subsequent transition to blood-stage infection.

TABLE 3 | The comparative analysis of different LS humanized mice to study inflammatory diseases, (Adapted and modified from Vaughan et al. (147).

		Alb-uPA SCID (Beige)	FAH$^{-/-}$Rag2$^{-/-}$IL2Rγ^{null} (FRG)	TK/NOG	HLA-DR4.RagKO.IL2Rγ cKO.NOD (DRAG)
Liver Injury	Mutation	uPA (urokinase-type plasminogen activator) overexpression	Fumarylacetoacetate hydrolase deficiency	Herpes simplex virus type 1 thymidine kinase (HSVtk) transgene expression	NRG mice chimeric with human-mouse class II transgenes encoding the HLA-DR4 genotype fused to the *I-E*^{d} MHC class II molecule
	Occurrence	At birth	At birth and increased by the NTBC treatment	On day 7 and 5 prio to transplantation, liver cells treated with GCV and maintain without any exogenous drug pressure	At birth
Transplantation age		Within 3 weeks of post-birth	Any (adult)	Adult 8-week-old	Four months of post infusion of human HSC
Human Chimerism		up to 100%	up to 90%	more than 90%	High (human cells and HSCs) but human RBCs were less (0.2–1%)
Throughput		Low	Medium	High	Medium
Additional challenges		Continuing and progressive damage to liver parenchymal stage, poor breeding efficiency, renal disease	Development liver carcinomas, under drug exposure for longer duration	No systemic morbidity	Less number of human hepatocytes developments, develop a functional human immune system, development of mouse monocytes
Plasmodium falciparum	Sporozoite infection	Yes	Yes	Yes	Yes
	Liver stage development				
	Early	✓	✓	✓	✓
	Mid	✓	✓	✓	✓
	Late (merozoite release)	✓	✓	✓	✓
	Liver-to-blood stage transition	No	Yes	Yes	Yes
Erythrocyte co-engraftment possible		No	No	Yes	Yes (low)
Unique advantage		No	No	Additional GCV treatment is used twice only prior to huHep transplantation	Fusion with HSC develop human hepatocytes, kupffer cells, liver endothelial cells and erythrocytes, Sustain complete life cycle of *P. falciparum* without exogenous addition of human hepatocytes/RBCs
Stability and reproducibility of humanized liver		human chimerism can only be achieved in homozygous SCID/ Alb-uPA immunodeficient recipients	Long life span through NTBC drug treatment, but other associated complications are seen	Over the 8 months of pro-long period without any drug pressure	Human RBCs can detect for up to 4 months
References		(147)	(147)	(6, 148)	(146)

NTBC, 2-(2-nitro-4-(trifluoromethyl)benzoyl)cyclohexane-1,3-dione; GCV, ganciclovir.

human cells/tissues. A major contribution of these mouse model(s) is to study the erythrocytic stage and liver stage infection of *P. falciparum*. We believe an ideal humanized mouse model will be instrumental to study novel drugs and their targets, assessing the immunogenicity of novel candidate vaccines. Higher immunosuppression and novel transplantation strategies are indeed needed to create a small laboratory, straightforward and reproducible humanized mouse model. This review will help the development of new methods to create a humanized mouse with stable human cell transplantation and repopulation, to study systemic inflammatory diseases.

AUTHOR CONTRIBUTIONS

RT conceived the idea of the study, carried out all experiments related to mouse humanization and modeling *P. falciparum* in humanized mice and wrote the manuscript. NT and RD helped format and write the manuscript. RE reviewed the manuscript. SP provided logistics. PT helped in the preparations of figures and tables and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02550/full#supplementary-material

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REVIEW
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Parasite Recognition and Signaling Mechanisms in Innate Immune Responses to Malaria

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Malaria caused by the *Plasmodium* family of parasites, especially *P.falciparum* and *P. vivax*, is a major health problem in many countries in the tropical and subtropical regions of the world. The disease presents a wide array of systemic clinical conditions and several life-threatening organ pathologies, including the dreaded cerebral malaria. Like many other infectious diseases, malaria is an inflammatory response-driven disease, and positive outcomes to infection depend on finely tuned regulation of immune responses that efficiently clear parasites and allow protective immunity to develop. Immune responses initiated by the innate immune system in response to parasites play key roles both in protective immunity development and pathogenesis. Initial pro-inflammatory responses are essential for clearing infection by promoting appropriate cell-mediated and humoral immunity. However, elevated and prolonged pro-inflammatory responses owing to inappropriate cellular programming contribute to disease conditions. A comprehensive knowledge of the molecular and cellular mechanisms that initiate immune responses and how these responses contribute to protective immunity development or pathogenesis is important for developing effective therapeutics and/or a vaccine. Historically, in efforts to develop a vaccine, immunity to malaria was extensively studied in the context of identifying protective humoral responses, targeting proteins involved in parasite invasion or clearance. The innate immune response was thought to be non-specific. However, during the past two decades, there has been a significant progress in understanding the molecular and cellular mechanisms of host-parasite interactions and the associated signaling in immune responses to malaria. Malaria infection occurs at two stages, initially in the liver through the bite of a mosquito, carrying sporozoites, and subsequently, in the blood through the invasion of red blood cells by merozoites released from the infected hepatocytes. Soon after infection, both the liver and blood stage parasites are sensed by various receptors of the host innate immune system resulting in the activation of signaling pathways and production of cytokines and chemokines. These immune responses play crucial roles in clearing parasites and regulating adaptive immunity. Here, we summarize the knowledge on molecular mechanisms that underlie the innate immune responses to malaria infection.

Keywords: malaria, immunostimulatory factors, host receptors, signaling mechanisms, innate immune responses, protective immunity, pathogenesis

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BACKGROUND

Malaria is a widespread infectious disease that is prevalent in most tropical regions of the world (1–4). About half of the world population is at risk of contracting malaria. World Health Organization has reported an estimated 216 million malaria clinical cases and about 445,000 deaths during 2016 (1). Besides huge health burden and mortality, malaria morbidity is a substantial hindrance to socio-economic development in endemic areas due to the loss of man power (5, 6). The disease is caused by protozoan parasites of the genus *Plasmodium*. Five parasite species infect humans that include *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* (7–9). However, most malaria infections are caused by *P. falciparum* and *P. vivax*, and infections by the other three species are relatively rare. Several parasite species, including *P. berghei*, *P. yoelii*, *P. chabaudi,* and *P. vinckei*, infect rodents, but not humans (10). Different strains of rodent parasites used in laboratories show distinct growth rates, and infected mice exhibit a range of immunological and pathological conditions, resembling a wide spectrum of pathophysiologic conditions of human malaria infection. As such, mice infected with different parasite strains are useful models to study distinct systemic and organ-specific clinical conditions of human malaria.

Malaria is a highly complex disease that displays a wide variety of pathological conditions. At the early stages, malaria infection presents a number of systemic clinical conditions, including the characteristic periodic fever, chills, headache, dizziness, malaise, abdominal discomfort, nausea, and muscle and joint aches (11, 12). As the infection progresses and parasite biomass increases, pathogenic processes follow, resulting in severe anemia, blood acidosis, splenomegaly and hepatomegaly, acute respiratory distress syndrome, and several other clinical conditions. In the case of *P. falciparum*, the infected red blood cells (IRBCs) bind to certain cell surface proteins of vascular endothelia, including CD36, intracellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), and endothelial protein receptor (EPCR) (4, 13–20). These binding events allow parasites to sequester in organs, such as brain, lungs, liver, intestine, dermal tissues, and placenta, thereby avoiding splenic clearance. Parasite sequestration contributes to single and multiorgan fatal pathologic conditions, including cerebral malaria, and renal, liver and lung dysfunction and failure. In pregnant women, *P. falciparum* sequesters in the placenta through the binding of IRBCs to chondroitin 4-sulfate in the intervillous space and on the syncytiotrophoblast cell surface (21–23). This process contributes to pregnancy-associated malaria, characterized by a number of clinical conditions, including low birth weight, abortion, and death in the baby and the mother (24). The binding of IRBCs to the endothelial cell surface proteins in the microvasculature of vital organs and chondroitin 4-sulfate in the placenta is mediated by a family of antigenically variant parasite proteins encoded by about 60 *var* genes (15, 16, 25). These proteins are collectively called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 confers virulence to *P. falciparum* through IRBC binding to endothelial cells in various organs contributing to microvascular plugging and hypoxia. In addition, IRBC-binding and parasite accumulation amplify inflammatory responses locally, leading to immune cell infiltration, endothelial damage, and organ dysfunction and failure. *P. vivax* lacks PfEMP1 ortholog (26) and thus, *P. vivax* is less virulent compared to *P. falciparum*. However, in recent years, *P. vivax* is becoming increasingly virulent, causing severe malaria in significant numbers (11, 27–30). Although, the underlying reasons in *P. vivax* causing severe illnesses remain unclear, drug resistance and changes in genetic makeup appear to be significant factors (31, 32).

Innate immune responses that are initiated in response to malaria infection play key roles both in the development of protective immunity and pathogenesis (14, 33–38). Early pro-inflammatory responses regulate antiparasitic Th1 development and promote effector cell function for efficiently clearing infections. Usually, as infection progresses, pro-inflammatory responses are gradually downregulated with parallel increase in anti-inflammatory responses (39). Generally, this leads to Th2 development, resulting in balanced pro-/anti-inflammatory and Th1/Th2 responses and resistance against pathogenesis (35, 39–42). However, this is not always the case. Depending upon the host-parasite interaction dynamics, factors such as parasite sequestration and alterations in host genetics, pro-inflammatory responses may be overly upregulated, resulting in systemic and organ-related severe illnesses.

During the past two decades, there has been a substantial progress in our understanding of the host receptors that sense parasite factors involved in inducing immune responses and the associated signaling pathways (43–47). A substantial progress has also been made in our understanding of parasite immunostimulatory factors that are the targets of host receptors (43–47). However, much remained to be learned on the spectrum of molecular and cellular processes and immune responses that are initiated upon parasite sensing. Development of protective immunity to malaria requires repetitive infection over a period of time (48, 49). Information is limited as to whether and how the innate immune responses contribute to the inefficient development of protective immunity to malaria. In addition to signaling initiated by specific sensing of parasite factors, signals

Abbreviations: IRBCs, infected red blood cells; DC, dendritic cell; Mφ, macrophage; NK cells, natural killer cells; NKT cells, NK T cells; GPI, glycosylphosphatidylinositol; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; ICAM-1, intracellular adhesion molecule 1; VCAM-1, vascular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen-1; EPCR, endothelial protein receptor; Th, helper T cells; IFNαR, type I IFN-α receptor; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; PRRs, pathogen recognition receptors; TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; MAL, MyD88 adapter-like; IRAK, IL-1 receptor-associated kinase; TRAF6, tumor necrosis factor receptor-associated factor 6; TRIF, TIR (Toll/IL-1 receptor) domain-containing adaptor inducing IFN-β; TRAM, TRIF-related adaptor molecule; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; NF-κB, nuclear factor κB; TBK1, TRAF family member-associated NF-κB activator (TANK)-binding kinase 1; cGAS, cyclic GMP-AMP synthase; STING, stimulator of IFN genes; MDA5, melanoma differentiation-associated gene 5; MVAS, mitochondrial antiviral-signaling protein; IRF, IFN regulatory factor; AIM2, absent in melanoma 2; NOD, nucleotide-binding oligomerization domain; NLR, (NOD)-like receptor; LLR, leucine-rich repeat kinase-like protein; NLRP, NOD- LRR- and pyrin domain-containing protein.

TABLE 1 | Prevalence and features of human blood stage malaria parasites.

Parasite species	Prevalence	Erythrocyte stage life cycle duration	Merozoite numbers in matured IRBCs	Cytoadherence capacity and organs where adherence occurs	Frequency of causing severe illnesses and fatality	References
P. falciparum	Worldwide (high prevalence in Africa)	48 h	8–24	Strong; in most organs, including skin, intestine and placenta	Often causes severe illnesses, highly fatal	(53)
P. vivax	Asia, Latin America, some parts of Africa	48 h	12–18	Weak; mostly in lungs	Mostly uncomplicated malaria, occasionally fatal	(53)
P. ovale	Africa, Western pacific islands	48 h	8–16	Absent	Mostly uncomplicated malaria, fatality is rare	(53, 54)
P. malariae	Worldwide	72 h	6–12	Absent	Mostly uncomplicated malaria, fatality is rare	(53, 54)
P. knowlesi	Southeast Asia	24 h	Up to 16	Weak; in lungs, and likely brain and other organs	Uncomplicated to severe illnesses (60–70% of infected cases develop ARDS[a])	(8, 9, 55)

[a]*ARDS, acute respiratory distress syndrome.*

induced by processes such as phagocytosis and cytoadherence also contribute; information is also limited as to whether and to what extent these signaling contribute to the overall innate immune responses. In this review, we are providing an overview of the available information on the molecular and cellular mechanisms of parasite-host interactions that involved in innate immune responses to malaria.

MALARIA INFECTION

Malaria infection begins with the entry of sporozoite form of parasites when infected mosquitoes inject saliva during blood meal (7, 46, 50–52). A substantial number of injected sporozoites is unable to enter the blood stream and stuck in the dermis, and these parasites are removed likely by the resident macrophages (Mφs). Those sporozoites that enter the blood stream target liver, where they exclusively infect hepatocytes. In hepatocytes, parasites reside inside parasitophorous vacuole formed during invasion and develop into merozoites over a period of 7–10 days in humans and 2 days in rodents, vastly expanding hepatocyte size. Upon parasite maturation, each infected hepatocyte releases 10,000–30,000 merozoites into the blood stream (46, 50–52). This period of cell cycles represents the first stage of malaria infection, referred to as the liver stage or the tissue stage infection. The merozoites released from the matured infected hepatocytes are called exo-erythrocytic merozoites. These merozoites do not infect hepatocytes, but instead exclusively invade red blood cells, and reside inside parasitophorous vacuole. Soon after invasion, parasites appear morphologically like rings inside IRBCs. Parasites then develop into early and late trophozoites, and finally undergo schizogony to form differentiated merozoites, which are released into the blood stream. Each matured erythrocytic stage schizont releases 8–24 merozoites (53) (**Table 1**), which can invade red blood cells. The parasite developmental process inside red blood cells occurs over a period of 24–72 h, depending on the *Plasmodium* species (**Table 1**). This cycle of invading red blood cells and parasite growth is called the blood stage infection. The repetitive erythrocytic cell cycles result in the exponential growth of parasites and if the growth is unchecked, most red blood cells are consumed, resulting in severe anemia and pathologies, and death. However, soon after infection, the innate immune system detects parasites at both the liver and blood stages, and responds by inducing pro-inflammatory cytokines and chemokines. The cytokines prime phagocytes for an efficient uptake and clearance, while chemokines help recruit effector cells to sites, where parasites are sequestered or accumulated, for effective infection clearance.

PATHOGEN SENSING MECHANISMS

Hosts respond to various infections by sensing certain evolutionarily conserved molecules (signature structures) of pathogens called pathogen-associated molecular patterns (PAMPs); these include bacterial LPS and peptidoglycan, fungal glucans, and microbial DNA and RNA, and even self-DNA under certain pathologic conditions (56–61). Host detects PAMPs through receptors called pathogen-recognition receptors (PRRs). Host also detects certain endogenous factors released during infection and thus can induce danger signaling. These factors are called danger-associated molecular patterns (DAMPs). Examples of DAMPs include high mobility box 1 (HMGB1), HSP70, SP100 family of proteins, and degraded hyaluronic acid (62–65). The innate immune system is equipped with a wide range of PAMP- and DAMP-recognizing PRRs. After recognition of PAMPs and DAMPs by PRRs, the innate immune cells are activated through the initiation of specific signaling pathways, producing cytokines and chemokines. PRRs are present at various cellular locations, including outer surface of plasma membrane, luminal surface of endosomal membrane, outer membrane of mitochondria, and cytosol (59, 60, 66). Prominent among transmembrane PRRs are toll-like receptors (TLRs) (56–59, 61); others transmembrane PRRs include, c-type lectin receptors, such as mannose- and galactose-binding proteins, and scavenger receptors, such as CD36, CD204, and MARCO (67). Examples of cytosolic sensors include dectin-1 that binds

fungal β-1,3-glucan, cyclic GMP-AMP synthase (cGAS, senses dsDNA), retinoic acid-inducible gene-I (RIG-I, respond to viral RNA), RIG-I-like receptors (RLRs), such as melanoma differentiation-associated gene 5 (MDA5) that respond to dsRNA, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs, senses bacterial peptidoglycan), and absent in melanoma 2 (AIM2, respond to dsDNA). Malaria parasites are sensed by several receptors, leading to cell activation and immune responses (see below). Several excellent reviewers that comprehensively discuss various host-pathogen interactions and signaling mechanisms are available (56–60, 62–66, 68–70).

MALARIAL PAMPs AND HOST PRRs

A notable feature of malaria infection is that the liver stage is clinically completely silent, that is, the host does not exhibit any symptoms of malaria (71–73). All the malaria clinical conditions and fatal illnesses are manifested during the blood stage infection (74). At both stages of infection, the host detects parasites immediately after infection and initiates innate immune responses. These responses are geared toward clearing the infection and shaping the development of protective adaptive immunity (14, 34, 44, 71–78). However, the complex parasite-host interaction dynamics are not always in favor of achieving this goal, but instead often result in dysregulated immune responses and uncontrolled parasite growth, leading to pathogenesis. Understanding the malaria parasite-host interaction dynamics that shape the parasite-specific immunity and the molecular and cellular processes that contribute to pathogenesis is important for developing suitable treatment strategies. Below, we summarize the advancements that have been made in identifying the malaria PAMPs, the cognate host PRRs, and the signaling events that lead to innate immune responses.

LIVER STAGE PARASITE SENSING

Since the liver stage malaria infection is clinically silent, it has long been thought that parasites inside hepatocytes grow undetected by the innate immune system. However, recent studies in *P. berghei*- and *P. yoelii*-infected mice show that, although parasites inside hepatocytes are shielded from recognition by Mφs and dendritic cells (DCs), the growing parasites are recognized by the cytosolic PRRs of hepatocytes,

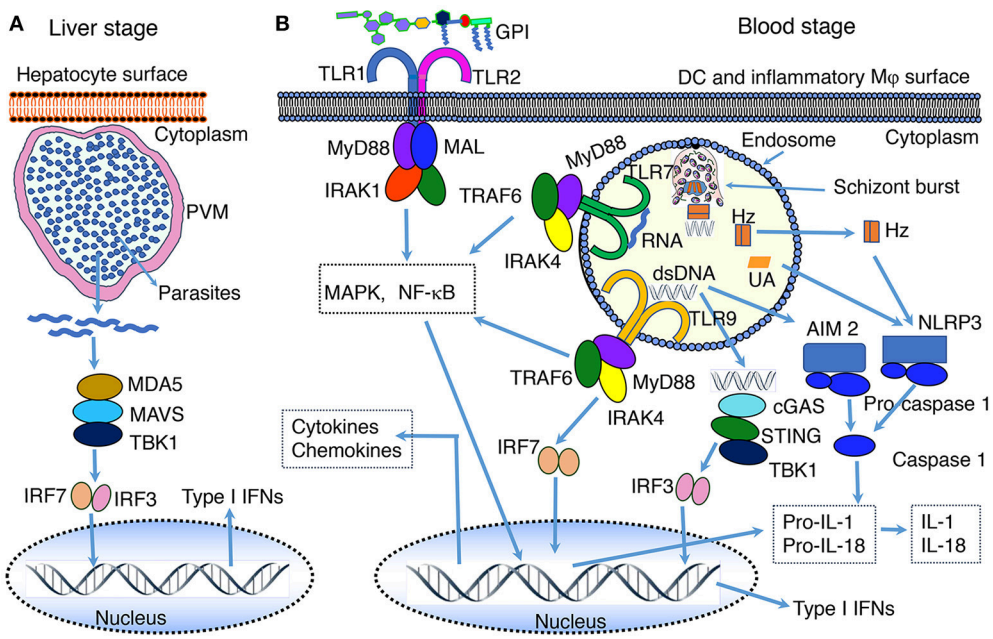

FIGURE 1 | PAMP-PRR interaction-induced signaling pathways. **(A)** RNA of the liver stage parasites growing inside hepatocytes is recognized by MDA5 leading to the activation of MAVS-TBK1-IRF3/IRF7 signaling and downstream production of type I IFNs. **(B)** At the blood stage infection, parasite DNA, RNA and GPI interact with, respectively, TLR9, TLR7, and TLR2, leading to the activation of primarily MAPK and NF-κB signaling pathways and downstream cytokine and chemokine responses. In the cytosol, similar to the liver stage parasite RNA, the blood stage parasite RNA is sensed by MDA5 leading to the activation of MAVS-TBK1-IRF3/IRF7 signaling (see **A**). However, this signaling seems induce the expression of SOCS1, which downregulate RNA-TLR7-induced type I IFN production (81). Parasite DNA in the cytosol is sensed by cGAS, resulting in the activation of STING-TBK1-IRF3 signaling and type I IFN response. Parasite DNA also activates AIM2 inflammasome, which cleaves pro-caspase 1 to activate caspase 1. Hemozoin (Hz) and uric acid (UA) induce danger signaling, activating NLRP3 inflammasome and the cleavage of pro-caspase 1 to activate caspase 1. Parasites have also been reported to activate NLRP12 inflammasome through unidentified interaction, leading to the cleavage of pro-caspase 1 to activate caspase 1 (44, 82). It appears that microparticles released from IRBCs and heme produced during infection activate TLR4 signaling (83, 84). Ligands bind to TLR4 homodimer through the cooperation of accessory proteins CD14 and MD2, leading to MAPK, NF-κB and TRIF-TBK1-IRF3 signaling. Note: the diagram depicts a simplified version of indicated signaling pathways and additional details can be found in review articles (44, 56–70). The abbreviations are defined in footnote in page 1.

initiating antiparasitic type I IFN response (79, 80). The parasites in infected hepatocytes are detected through the interaction of parasite RNA with RIG-I family of proteins homolog called melanoma differentiation-associated protein 5 (MDA5), but not by RIG-I, leading to the activation of MDA5-MAVS-IRF3/IRF7 signaling axis and downstream production of type I IFN (79, 80) (**Figure 1**). This signaling event results in an array of type I IFN receptor (IFNαR)-mediated innate immune responses, which include: (i) expression of interferon-stimulated genes by hepatocytes; (ii) production of chemokines by hepatocytes, and chemotaxis-mediated recruitment of Mφs, neutrophils and lymphocytes to the proximity of infected hepatocytes; (iii) production of IFN-γ and chemokines by NK and NKT cells, which are abundantly present in the liver; (iv) infiltration of NK and NKT cells to the liver; (v) CD1d-resitrcted elimination of infected hepatocytes by NKT cells (79, 80). Since parasites reside inside parasitophorous vacuole, it appears that parasite RNA is exported to the cytosol, but not to phagolysosomes. It is unlikely that cytosolic RNA can enter endosomes and moreover hepatocytes may not express significant levels of TLR7. Thus, it appears that cytosolic sensors are the only PRRs that interact with parasite factors in infected hepatocytes. These findings represent a significant advancement in our understanding of parasite recognition mechanisms involved in innate immune responses to the liver stage malaria infection. However, it remains unclear if parasite DNA is exported to the cytosol, where it can be sensed by cytosolic nucleic acid sensors. DNA is a prominent immunostimulatory PAMP of the blood stage malaria parasites and the AT-rich stem loops of parasite DNA can induce the production of type I IFN through sensing in the cytosol (44, 60). If the liver stage parasite DNA has no role in type I IFN response, then DNA is either not exported to the cytosol of hepatocytes or non-stimulatory.

BLOOD STAGE PARASITE SENSING

As mentioned earlier, the blood stage infection accounts for all the pathological conditions of malaria (74). In infected non-immune people, *P. falciparum* parasites multiply rapidly through the release of large numbers of merozoites by matured schizonts to the blood circulation every 48 h (53, 85). Some of the released merozoites invade erythrocytes and the reminders become dead and are likely targeted by Mφs and DCs. In addition, the burst of schizont stage erythrocytes releases large amounts of parasite's digestive vacuoles containing hemozoin and other waste products. The blood stage parasites grow synchronously by adapting to daily rhythm of systemic TNF-α production and the level of glucose in the blood (86). Thus, parasites develop at similar rates, releasing merozoites, digestive vacuoles and other contents more or less at the same time point (86). This leads to peak concentrations of parasite stimulatory components, to which the innate immune system potently responds and induces the production of pro-inflammatory cytokines and chemokines at high levels. This periodic elicitation of strong inflammatory responses within a narrow window of time period after each erythrocytic cell cycle is responsible for the characteristic periodicity of malaria paroxysms, including periodic recurrent of peak levels of fever, chills, headache, shock, and malaise. Several malaria parasite PAMPs have been identified, and the mechanisms by which they are sensed and the associated signaling pathways and immune responses have been delineated (44, 46, 73, 87). This body of information represents a substantial progress in our understanding of malaria innate immunity. Below we describe the current knowledge on malaria parasite PAMP and host PRR interaction mechanisms involved in innate immune responses.

MALARIAL PAMPs

GPI

P. falciparum glycosylphosphatidylinositol (GPI) is the first factor that was identified as a malaria parasite PAMP (88). Structurally, parasite GPI comprises a heterogenous group of molecules consisting of triacylated phosphatidylinositol linked to the glucosamine moiety of glycan having four mannose residues and a glucosamine residue (89–91). The structural heterogeneity of malaria GPI is due to the variation in length and level of unsaturation in acyl residues present at different positions of the phosphatidylinositol moiety. This structural heterogeneity in the lipid moieties has no bearing on immune response-inducing activity of GPI. This is evident from the fact that the *sn-2 lyso* GPI, obtained by the removal of acyl moiety at the *sn-2* position of parasite GPI, could efficiently induce cytokine responses similar to the unmodified parasite GPI. The biosynthesis of GPI is essential for the survival of parasites as GPI anchors several proteins of merozoites to the plasma membrane that are involved in erythrocyte invasion (92–94). In the absence of GPI anchoring, these proteins are not expressed on the surface and hence merozoites cannot invade erythrocytes. Malaria parasites synthesize GPI in several folds excess over the actual amounts needed for anchoring proteins to the surface of merozoites and thus, significant amounts of GPI remain not linked to proteins (90). The GPI molecules that are not linked to proteins are exposed on the cell surface and thus are likely targeted by the innate immune system.

The identification that parasite GPI is a malaria inflammatory response-inducing pathogenicity factor was based on the observation that the purified parasite GPI could induce strong pro-inflammatory responses by Mφs (88). When administered to mice, GPI induced symptoms that resembled the systemic clinical conditions of malaria, including pyrexia, cachexia, hypoglycemia, and TNF-α-induced sepsis. In several subsequent studies, GPI was shown to induce a wide range of immune responses, including the production of TNF-α and IL-1 by Mφs, expression of nitric oxide synthase by Mφs and endothelial cells, and the expression of ICAM-1, VCAM-1, and E-selectin by leukocytes and endothelial cells through the activation of several signaling events (95–97). Consistent with the property of GPI in inducing malaria-like symptoms, immunization of mice with a synthetic glycan portion of GPI produced anti-GPI antibodies, and immunized mice infected with *P. berghei* ANKA, an experimental cerebral malaria model, were protected from cerebral malaria

(98). Further, the presence of anti-GPI antibodies in people in malaria endemic was associated with a significant protective immunity against malaria illnesses (99, 100).

Subsequent studies have shown that *P. falciparum* GPI activates Mφs through the induction of an outside-in signaling, without binding to plasma membrane or being internalized by the cells, but instead by recognition through weak interactions on the cell surface (101). The intact structure of GPI is essential for bioactivity as neither the carbohydrate moiety nor the triacylated phosphatidylinositol lipid portion can induce immune responses. Further studies have shown that the parasite GPI induced production of pro-inflammatory cytokines by Mφs occurs through recognition mainly by TLR2-TLR1 heterodimer and to a much lesser extent by TLR4 (102–105); **Figure 1** and **Table 2**. Sensing of GPI by TLR2-TLR1 leads to the activation of ERK, p38, JNK MAPK, and NF-κB signaling pathways, which differentially contribute to the production of various inflammatory mediators (103). Thus, malaria parasite GPI is mainly a TLR2-activating PAMP.

DNA

A large body of accumulated data over the past two decades demonstrates that microbial (bacteria, viruses, and parasites) DNA, and self-DNA in some pathological situations, function as PAMP and are recognized by TLR9 in endosomes and by DNA sensors in the cytosol (56–60). In the case of malaria, the first demonstration that TLR9 senses malaria parasites and induces immune responses was by Pinchyangukul et al. (123). They showed that a soluble component in the schizont extract of *P. falciparum* that was heat labile and precipitable with ammonium sulfate (the description agrees with the active component being protein-DNA complex) induces cytokine and chemokine responses by human plasmacytoid DCs (pDCs) and mouse DCs through the activation of TLR9-MyD88 signaling pathway. Subsequently, it was shown that the TLR9 signaling-inducing malaria factor is DNA (107, 108, 124). TLR9 specifically recognizes the unmethylated CpG motifs of DNA (68, 69). The genomic DNA of *P. falciparum* and *P. vivax* contain, respectively, ∼300 and ∼2,000 CpG motifs (109). The higher content of CpG motifs is likely responsible for the strong fever-inducing ability of *P. vivax* compared to *P. falciparum* (125).

Malaria parasite DNA enters the endosomes of the innate immune cells, such as Mφs and DCs, through phagocytic uptake of IRBCs, merozoites, the nuclear material of parasites, DNA-protein-hemozoin complex, and DNA-containing immune complexes (107, 108, 124, 126). The endosomes are then fused to lysosomes to form phagolysosomes. In the acidic environment of phagolysosomes, DNA is released and recognized by TLR9, leading to the activation of MAPK and NF-κB signaling pathways and cytokine and chemokine responses (**Figure 1** and **Table 2**). In addition to TLR9, parasite DNA is recognized by cytosolic DNA sensors upon the release of phagolysosomal contents into the cytosol. In the cytosol, several distinct cytosolic sensors can potentially recognize DNA (**Figure 1**). Thus far, it has been demonstrated that two cytosolic PRRs sense parasite DNA: (i) cGAS sensing dsDNA and inducing the activation of STING-TBK1-IRF3 signaling and downstream production of type I IFNs (44, 110), and (ii) AIM2 recognizing dsDNA, resulting in the activation of inflammasome and caspase 1. The activated caspase 1 converts pro forms of IL-1 and IL-18 into active IL-1 and IL-18. Robust production of IL-1 and IL-18 in response to malaria infection requires, in addition to inflammasome signaling, TLR (mainly TLR9 and TLR7) mediated production of pro-IL-1 and pro-IL-18. *P. falciparum* genomic DNA contains >80% AT nucleotides. The AT-rich motifs of parasite DNA form loop structures, which have been shown to induce type I IFN response through STING-TBK1-IRF3 signaling (109). In some viruses, the AT-rich loop motifs of DNA are transcribed by RNA polymerase III to form double-stranded RNA containing 5′-triphosphate that induces type I IFNs through RIG-I-MAVS-IRF3 signaling (110). However, in the case of *P. falciparum* DNA, pol III-dependent recognition of AT-rich motifs of DNA seems to be not involved in cytosolic sensing as deficiency in pol III had no effect on immune responses to the parasite DNA (109).

RNA

The innate immune system senses RNA of both the liver and the blood stage parasites. In the liver stage, as described in the section Liver Stage Parasite Sensing above, RNA is recognized exclusively in the cytosol by MDA5 (**Figure 1**) (79, 80). In contrast, in the blood stage infection, mouse parasite RNA is recognized by TLR7 in phagolysosomes of DCs, leading to type I IFN production (**Figure 1**). In fact, studies in a mouse model of *P. chabaudi*-infection showed that type I IFN production is the earliest cytokine response (24 h postinfection) during the blood stage malaria infection (127). Parasite RNA enters endosomes of Mφs and DCs upon the uptake of parasites/parasite components, inducing type I IFN response through TLR7 signaling (81, 106, 128). Type I IFNs thus produced prominently contribute to the upregulation of pro-inflammatory cytokines, such as IFN-γ and IL-12, during the early stages of infection (106). RNA of the blood stage mouse parasites is also sensed by cytosolic MDA5, leading to type I IFN production (81).

While it is clear that RNA induced TLR7 signaling plays an important role in early type I IFN production in mouse malaria, it remains unclear whether or to what extent RNA of human parasites is able to induce type I IFN response. This is because, although RNA of human malaria parasite *P. falciparum* has been reported to induce cytokine responses through TLR7 recognition, the reported activity appears to be very low (106). Further studies are needed to determine whether or to what extent *P. falciparum* RNA is immunostimulatory.

MALARIAL DANGER-ASSOCIATED MOLECULAR PATTERNS (DAMPs)

Hemozoin

Hemozoin is a hydrophobic, crystalline insoluble polymer of heme formed during the digestion of hemoglobin by parasites in the digestive vacuoles to use the released amino acid as a food source (45, 129–131). In parasites, hemozoin is associated with lipids and proteins, and is released into the blood circulation when merozoites are egressed from matured schizonts. Hemozoin by itself is an inert material and appears

TABLE 2 | Innate sensing of malaria parasites and signaling mechanisms.

PRRs (host receptors)	PRR cellular location	PAMPs and DAPMs	Signaling pathway	References
TLRs				
TLR1,2 dimer	Cell surface	GPI	MAPK, NF-κB	(43, 89, 103, 104)
TLR4	Cell surface	GPI, heme, IRBC micro-particles	MAPK, TRIF, NF-κB	(43, 44, 83, 84)
TLR7	Endosome	RNA	MAPK, NF-κB	(106)
TLR9	Endosome	DNA	MAPK, NF-κB	(44, 107, 108)
CYTOSOLIC NUCLEIC ACID SENSORS				
MDA5	Cytosol	RNA	MAVS-TBK1-IRF3/IRF7	(79–81)
cGAS	Cytosol	DNA	STING-TBK1-IRF3/IRF7	(44, 109, 110)
AIM2	Cytosol	DNA	NLRP3 inflammasome	(44, 109, 111)
DANGER SIGNALING				
-	Cytosol	Hemozoin	NLRP3 inflammasome	(45, 111–114)
-	Cytosol	Uric acid	NLRP3 inflammasome	(114–116)
-	Cytosol	Unidentified factor	NLRP12 and NLRP4 inflammasome	(44, 82)
PHAGOCYTIC RECEPTORS				
CD36	Cell surface	PfEMP1, and unidentified ligand(s)	Src/Syk, MAPK	(117–120)
ADHESION RECEPTOR				
ICAM-1	Cell surface	PfEMP1	Src-PI3K-Akt, Rho, MAPK?	(121, 122)
EPCR	Cell surface	PfEMP1	Not known	

to have no specific receptor for recognition, but it influences the innate immune responses to malaria parasites in several ways; reviewed in Olivier et al. (45). Studies have reported that hemozoin is a carrier of malaria parasite DNA into endosomes for TLR9 recognition (107). Although how and where parasite DNA associates with hemozoin have not been specifically studied *in vivo*, there exist several possibilities. Malaria merozoites egressed from the matured schizonts have a half-life of <5 min (132), and many merozoites cannot invade red blood cells within this short period time. These uninvaded merozoites are likely to be lysed, releasing DNA that may complex with hemozoin *via* the associated proteins. Additionally, Mφs and neutrophils undergo apoptotic death after ingesting IRBCs (133), releasing the degraded parasite materials, including DNA, RNA, hemozoin and other components. The released DNA can complex with hemozoin. However, hemozoin is not obligatory for the entry of parasite DNA into endosomes. The parasite nuclear material, DNA containing immune complexes, merozoites, and IRBCs are also taken up by phagocytic cells (107, 108, 124, 126). It is difficult to quantify *in vivo* the extent to which DNA enters endosomes through phagocytic uptake of infected erythrocytes, merozoites and nuclear materials compared to hemozoin-bound DNA in inducing immune responses.

Since hemozoin is an inert, indigestible material, it curtails the ability of Mφs to induce innate immune responses to malaria. Upon uptake of IRBCs or hemozoin, Mφs become immunosuppressive and dysfunctional due to damages caused by oxidative burst (134). Monocytes and Mφs that ingest parasite IRBCs undergo apoptotic death with little or negligible release of cytokines, although cytokines are expressed to certain extent (133). Phagocytic internalization of large amounts of whole parasites and non-digestible hemozoin renders Mφs nonfunctional because of phagolysosomal acidification and apoptotic death (133, 135). Hemozoin is known to inhibit the differentiation and maturation of human monocyte-derived DCs as well (136, 137). However, certain subsets of inflammatory Mφs, such as spleen red pulp Mφs, $CD169^+$ Mφs, and $CD16^+$ monocytes produce cytokines in response to malaria parasites (126–128). It is not known whether hemozoin significantly alters the capacity of these cells to produce cytokines and chemokines.

The effect of hemozoin on DC function remains unclear, reviewed in Wykes and Good (138). A previous study has shown that hemozoin-internalized DCs localized in T cell areas of the spleen in *P. chabaudi*-infected mice and that T cell activated by these DCs lacked effector function (139). These results suggest that hemozoin considerably compromises DC function. However, in a later study, DCs efficiently produced DNA-TLR9 signaling-induced cytokines in response to DNA bound to natural hemozoin and thus it was suggested that hemozoin enhances the activity of DNA by facilitating its entry to endosomes (107). It remains unclear whether this is the case in infected host. On the other hand, it has been shown that subsequent to the uptake of parasite components and the activation of TLR7 and TLR9 signaling, hemozoin destabilizes phagolysosomal membrane, leading to the release of nucleic acid and hemozoin into the cytosol (111). The released parasite components are sensed by cytosolic PRRs, leading to several signaling pathways (44, 81, 111–113), including: (i) cGAS-STING-TBK1-IRF3 signaling by dsDNA; (ii) MDA5-MAVS-TBK1-IRF3 signaling by RNA; (iii) STING signaling mediated

by AT-rich motifs through sensing by an unidentified receptor; (iv) AIM2 inflammasome signaling induced by parasite DNA, and (v) NLRP12 inflammasome activation *via* an unidentified mechanism (82). In addition, hemozoin also activates NLRP3 inflammasome (45, 111–113); **Figure 1** and **Table 2**. The activation of NLRP3 inflammasome by malarial hemozoin is mediated through the activation of Lyn and Syk tyrosine kinases (45). Synthetic hemozoin also activates Lyn and Syk signaling and, depending on the morphology and particle size, induces distinct immune response. It appears that hemozoin has no specific PRR, but it induces a danger signal (112, 113); hence, hemozoin is a DAPM. While nucleic acid-TLR-mediated signaling results in the synthesis of large amounts of pro-IL-1, inflammasome signaling and activation of caspase 1 lead to the cleavage of pro-IL-1 to the active, fever-inducing IL-1. Thus, hemozoin plays a significant role in the production of IL-1, contributing to fever induction in infected people. Besides hemozoin, parasite biomass, and purified merozoites that are devoid of hemozoin can potentially activate inflammasome and caspase 1 as it is known that even inert materials such as alum, asbestos, silica, uric acid crystals and cholesterol particles, activate NLRP3 inflammasome (45).

Uric Acid

Uric acid is the final oxidation product of purine metabolism and is released in large amounts by dying cells. Under physiological conditions, uric acid exists as a monoionic urate and forms insoluble monourate crystal (140). Uric acid is an important antioxidant in plasma and can induce protective anti-inflammatory responses in vascular and other diseases (141, 142). However, excessive formation of uric acid promotes pathogenic conditions, such as severe and chronic inflammatory arthritis, gout, and certain metabolic syndromes (141, 142). The pathogenic role of uric acid is due to the activation of NLRP3 inflammasome, which results in caspase 1 activation and conversion of pro-IL-1 into active IL-1 (114–116); **Figure 1** and **Table 2**. During malaria, a large number of parasite-ingested Mφs, neutrophils and other immune cells die, which likely release high levels of uric acid. Importantly, a significant amount of uric acid is formed during purine nucleotide metabolism by parasites and accumulates as a precipitate in IRBCs (143). Large amounts of hypoxanthine also accumulate in parasite IRBCs. The uric acid precipitate and hypoxanthine are released to the blood upon schizont burst. In the blood stream, hypoxanthine is oxidized to uric acid. In agreement with its pathogenic role, high levels of uric acid are found in the blood circulation of patients having severe malaria (144).

PHAGOCYTOSIS- AND ADHERENCE-INDUCED SIGNALING

Phagocytic uptake of parasite IRBCs, merozoites, hemozoin, and immune complexes by Mφs, neutrophils and DCs is an important process during malaria infection. Studies have shown that phagocytosis generally activates Src/Syk family of nonreceptor kinases, leading to the activation of a wide range of signaling pathways, including PKC, RAS-ERK, Ca_2^+, NF-κB signaling (145–147). These signaling events can integrate into TLR and inflammasome signaling, thereby modulating immune responses (148). Phagocytosis of malaria parasites and hemozoin suppresses immune function of Mφs and monocytes due to phagolysosomal acidification (133, 135). However, phagocytosis by DCs and inflammatory Mφs likely induces Src/Syk-mediated signaling, contributing to immune responses. The signaling induced by CD36-mediated phagocytosis and IRBC adherence in malaria immune responses are described below.

CD36 Signaling

CD36 is a multifunctional class B scavenger receptor that binds diverse types of ligands and pathogens, facilitating internalization by cells (67, 118, 149). Many cell types, including Mφs, DCs, platelets, and endothelial cells, express CD36. CD36-mediated phagocytosis of pathogens and pathogenic molecules activates Src/Syk kinases, leading to the activation of ERK, p38 and Jun members of the MAPK signaling pathways and NF-κB (67, 118, 119, 150) (**Figure 2**). Signals of these pathways synergize with TLR and inflammasome signaling, contributing to innate immune responses (119, 156). In malaria, CD36 plays several roles, including (i) mediating the sequestration of *P. falciparum* in vascular capillaries through the binding of adhesive PfEMP1 expressed on the surface of IRBCs, (ii) phagocytic uptake of parasites, and (iii) enhancing innate immune responses (120, 157–162). Studies have shown that CD36-induced MAPK signaling contributes to the production of TNF-α in mouse malaria infection and modulates parasite GPI-induced cytokine responses in mouse Mφs and human blood DCs (120, 161, 163). In *P. falciparum* endemic regions, single nucleotide polymorphisms in the *Cd36* gene have been linked to protection from cerebral and other severe malaria (164, 165). A more recent study in *P. yoelii*-infected mice demonstrated that CD36 significantly contributes to cytokine responses by innate immune cells, and the upregulation of MHCII expression, phagocytic activity, Th1 responses and expression of complement and Fc receptors (117). Overall, these responses lead to decreased parasite burden in infected host. It is possible that, similar to CD36, other scavenger receptors, such as MARCO modulate innate immunity to malaria to a certain extent.

EPCR Signaling

Endothelial protein C receptor (EPCR), which is also known as activated protein C (APC) receptor, is a transmembrane glycoprotein in vasculatures that binds protein C and promotes thrombin-thrombomodulin complex-mediated protein C activation (166). Under normal conditions, thrombomodulin on the endothelial surface binds thrombin and activates protein C to APC; this process is strongly promoted by EPCR. APC is detached from EPCR and inactivates blood coagulation factors Va and VIIIa, thereby exerting anticoagulant effect. APC binding to EPCR confers cytoprotective roles, such as antiapoptotic, anti-inflammatory and barrier stabilization responses (153–155). In malaria, EPCR binds certain members of PfEMP1 expressed on the surface of IRBCs and contributes to severe malaria (17, 167–169). Although the downstream

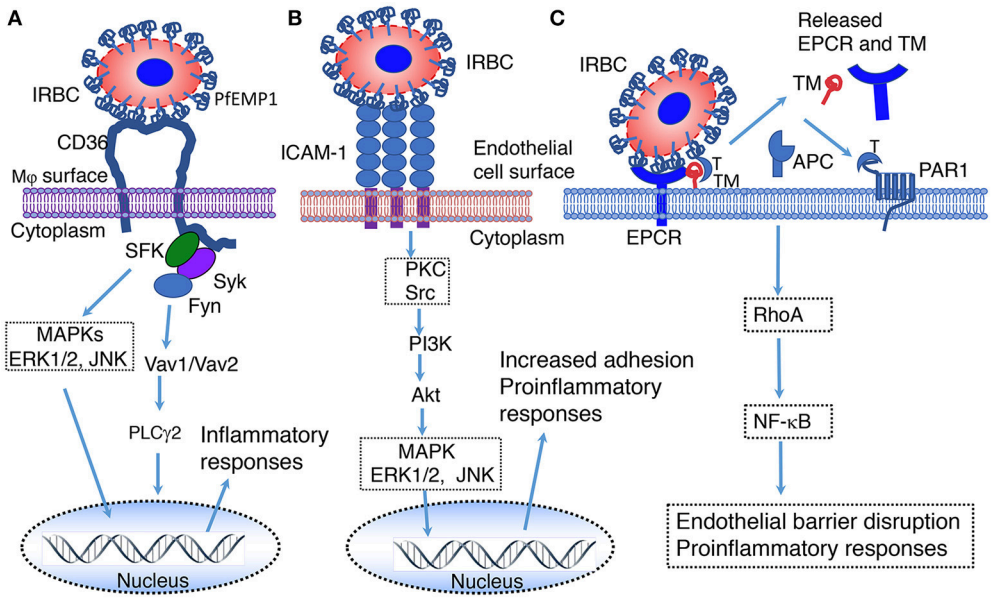

FIGURE 2 | Illustration of predicted pathways activated by the binding of PfEMP 1 expressed on the IRBC surface to CD36 **(A)**, ICAM-1 **(B)** and EPCR **(C)**. **(A)** Interaction of PfEMP1 on the surface of IRBC with CD36 on the surface of macrophages. SFK, Src family of protein tyrosine kinases; Syk, spleen tyrosine kinase; Fyn, proto-oncogene tyrosine protein kinase Fyn; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; JNK, c-Jun N-terminal kinase; Vav1/Vav2, Vav family of guanine nucleotide exchange factors; PLCγ2, phospholipase Cγ2 (151). **(B)** Interaction of PfEMP1 on the surface of IRBC with ICAM-1 on the surface of endothelial cells surface. PKC, protein kinase C; Src, proto-oncogene tyrosine-protein kinase Src; PI3K, Phosphatidyl inositol 3-kinase; Akt, RAC-alpha serine/threonine-protein kinase (152). **(C)** Interaction of EPCR on the surface of endothelial cells with PfEMP1 on the surface of IRBCs (153–155). EPCR, endothelial protein C receptor; TM, thrombomodulin; T, thrombin; APC, Activated protein C; PAR1, protease-activated receptor 1; RhoA, Ras homolog gene family, member A GTAase.

events of EPCR-PfEMP1 binding-mediated signaling remain to be understood, it appears that the binding results in the loss of EPCR and thrombomodulin from the endothelial cell surface by shedding, leading to decreased protein C activation and compromised EPCR-mediated protection (**Figure 2**). This may reduce anti-inflammatory responses and increase thrombin production, blood coagulation, pro-inflammatory responses, endothelial cell apoptosis, leading to the loss of barrier function. The cumulative effects of these responses may contribute to endothelial cell damage, promoting cerebral and other severe malaria illnesses.

ICAM-1 Signaling

ICAM-1 mediates the sequestration of *P. falciparum* in the brain through the binding of PfEMP1 on the surface of IRBCs to endothelial cells (15, 16, 25). The sequestration of parasites in vascular capillaries of the brain induces inflammatory responses, resulting in the infiltration of cytotoxic effector cells and endothelial barrier damage. These processes have been implicated in the development of cerebral malaria, but the experimental evidence is controversial. An earlier study has reported that interaction with ICAM-1 contributes to increased serum TNF-α in cerebral malaria model of *P. berghei* ANKA-infected mice and that ICAM-1 deficient mice survive >15 days compared to 6–8 days in wild type (WT) mice (170). However, a later study reported that ICAM-1 is dispensable for cerebral malaria pathogenesis (171). NK cells cross-talk with myeloid cells through LFA-1 binding to ICAM-1, producing IFN-γ (172). Similarly, T cells are also known to interact with endothelial cells through LFA-1 mediated binding to ICAM-1, producing IFN-γ. Studies have shown that, upon binding to its ligands, ICAM-1 is activated by phosphorylation, inducing Src-PI3K-Akt, Ca_2^+, Rho and MAPK signaling (121, 122) (**Figure 2**). It is likely that these signals contribute to immune responses to malaria; thus far this aspect has not been examined.

INNATE IMMUNE RESPONSES TO MALARIA

Innate Immune Responses at the Liver Stage Infection

In malaria, like in most pathogenic infections, the innate immune system functions as the first line of defense by controlling parasite growth and regulating the development of adaptive immunity (14, 75–77). As discussed in section Liver Stage Parasite Sensing, during the liver stage malaria infection, parasite-infected hepatocytes produce type I IFNs through cytosolic sensing of RNA. This cytokine response contributes to the killing of parasite-infected hepatocytes by NKT cells, exposing parasite components. The antigen-presenting cells, primarily DCs and inflammatory Mφs, can potentially recognize the exposed parasite factors. In addition, sporozoites injected by infected mosquitoes that could not enter the blood circulation

remain in dermis and die. In addition, some sporozoites that enter blood circulation may not invade hepatocytes and die. These dead parasites are likely sampled by DCs and inflammatory Mφs, leading to TLR- and inflammasome-mediated immune responses. The activated antigen-presenting cells have potential to modulate immune responses to the blood stage infection. However, because the parasite load in liver in natural infections is very low, the innate immune responses are likely to be also very low. Thus, the responses produced by antigen presenting cells against liver stage parasites may not exert a significant influence on immune responses induced by the blood stage parasites. However, in hyper endemic areas, repetitive infections in humans may induce immune tolerance in antigen-presenting cells that may modulate immunity to the blood stage infection to a certain extent.

Innate Immune Responses at the Blood Stage Infection

During the blood stage infection, unlike the liver stage, parasites grow exponentially through repetitive erythrocytic cycles, rapidly accumulating the biomass. This leads to an efficient induction of innate immune responses. Early during the blood stage infection, DCs and Mφs are key first responders of the innate immune system. However, as noted above, Mφs upon internalization of infected erythrocytes, merozoites or hemozoin become immunosuppressive and dysfunctional (133–137); unable to secrete cytokines and chemokines. It appears that the primary role of Mφs during the early stages of blood stage malaria infection is to control parasite growth through phagocytic clearance. In contrast to Mφs, DCs efficiently produce cytokines and chemokines in response malaria parasites, and effectively interact with cells of the innate and adaptive immune system. Thus, DCs play key roles in the initiation and regulation of innate and adaptive immunity to malaria. Also, it should be noted that some subsets of Mφs having certain features of DCs, such as $CD11c^+$ spleen red pulp Mφs and $CD169^+$ inflammatory Mφs, produce cytokines (127, 133, 173).

As noted in section RNA, during the blood stage malaria infection, type I IFNs are the earliest (24 h postinfection) cytokines produced through the activation of TLR7-MyD88 and IRF7 signaling (174). Early type I IFN response is produced primarily by the spleen red pulp Mφs and pDCs in *P. chabaudi*-infected mice (106, 127). Two recent studies by using lethal malaria model of *P. yoelii* YM infection, in which parasites grow rapidly to attain peak parasitemia of ∼80% by 6 days postinfection, have also showed TLR7-dependent peak levels of type I IFN production at 24–36 h postinfection (81, 128). This early cytokine response is mediated through the coordination of TLR7- and cytosolic sensing mechanisms: TLR7-MyD88-IRF7 signaling in endosomes, and DNA-cGAS-STING-TBK1-IRF3/IRF7 and RNA-MDA5-MAVS-TBK1-IRF3 signaling in the cytosol (81). It has been found that SOCS1 expressed in response to cytosolic nucleic acid sensor-mediated signaling significantly downregulates TLR7-mediated type I IFN response. As such, deficiency in SOCS1 results in markedly high levels of type I IFNs, providing resistance against high parasite burden-dependent lethality (81). Additionally, it has been shown that $CD169^+$ Mφs activated through STING-mediated signaling migrate to bone marrow, where they interact with pDCs to induce type I IFN production through TLR7-MyD88 signaling (128). Interaction of classical DCs with pDCs is also important in pDCs producing cytokine responses to malaria parasites (38, 108). It appears that Mφs and neutrophils that internalize parasites undergo pyroptosis (111), exposing parasite components, which are taken up by classical DCs and $CD169^+$ Mφs, and activated through STING-mediated cytosolic signaling. The early type I IFN response triggers the infiltration of immune cells to the blood that may subsequently localize to the spleen. In contrast, in *P. berghei* ANKA-infected mice, relatively high level of IFN-α was seen at 4 days postinfection with low or negligible IFN-α production during 1–3 days postinfection (175). Collectively, the above results indicate that different strains of malaria parasites differentially induce type I IFN. That is, the earliest type I IFN production (24–36 h postinfection) by *P. chabaudi* and *P. yoelii* is mediated mainly through parasite RNA-induced TLR7 signaling (81, 106, 128), whereas such RNA-mediated early type I IFN response is not readily apparent in *P. berghei* ANKA (175). The production of type I IFNs at later stages of *P. berghei* ANKA infection likely involves a different mechanism. In other studies, cGAS sensing of the AT-rich motifs of *P. falciparum* DNA in the cytosol could induce type I IFN production through the STING-TBK1-IRF3/IRF7 signaling pathway, independent of TLR9-MyD88 signaling (109, 110). This could be the mechanism through which *P. berghei* ANKA induces significant levels of type I IFN at 4 days pi (175).

Early type I IFN response to malaria infection contributes to the suppression of antiparasitic immunity and promotes cerebral and other severe malaria illnesses under certain situations. This is evident from the observations that deficiency in IFNαR in *P. yoelii* YM infection resulted in increased IFN-γ levels, significantly decreased parasitemia and resistant to parasite burden-dependent death (128). Also, in *P. berghei* ANKA-infected mice, which produce low levels of early type I IFN response but produce a significant amount at a somewhat later stage (4 days postinfection), deficiency in IFNαR or treatment with anti-IFNαR1 antibody resulted in increased IFN-γ production, increased number of IFN-γ-positive NK and $CD4^+$ T cells in the spleen, and significantly decreased parasitemia and protection from cerebral malaria (175, 176). From these results, it is evident that type I IFNs suppress the production of IFN-γ and thus anti-parasitic function under certain conditions. By contrast, in a different situation, high levels of type I IFN production at early stages of *P. yoelii* YM infection promoted antiparasitic immunity. In this case, blocking of SOCS1, a suppressor of cytokine signaling 1, expression resulted in high levels of type I IFN production, leading to increased IFN-γ production and reduced parasitemia, protecting mice from parasite burden-dependent death (81). Consistent with the observations of the latter study (81), daily treatment of *P. berghei* ANKA-infected mice with recombinant IFN-α, which to a certain extent resembles a situation of high levels of type I IFN production at early stage of infection, also significantly increased IFN-γ production by splenic $CD8^+$ T cells, markedly

reducing parasitemia and preventing cerebral malaria (177). The results of these two studies suggest that type I IFNs promote IFN-γ-dependent anti-parasitic immunity, providing resistance against severe malaria. On the other hand, treatment of *P. berghei* ANKA-infected mice with recombinant IFN-β resulted in reduced TNF-α and IFN-γ production, decreased expression of ICAM-1 and CXCL9 in the brain, reduced CXCR3 expression by T cells and T cell infiltration to the brain, thereby significantly preventing cerebral malaria and increasing mice survival (178). Thus, type I IFNs play contrasting roles in malaria in a context-dependent manner and also dependent on type I IFN isomeric composition.

Compared to the results of two studies outlined above (175, 176), wherein deficiency in IFNαR contributed to increased IFN-γ response and decreased parasitemia in *P. berghei* ANKA-infected mice, Palomo *et al.* observed contrasting immune responses (179), although in all these studies mice were protected from cerebral malaria. Palomo et al. found that, compared to infected WT mice, *P. berghei* ANKA-infected mice deficient in IFNαR had similar parasitemia, reduced expression of IFN-γ and granzyme B by T cells, decreased expression of T cell-attracting chemokine CXCL9, and low infiltration of $CXCR3^{+}$-expressing CD8 T cells to the brain. A notable difference in these studies is that the latter study used GFP knock-in transgenic parasite clone, which appeared to have slower growth rate than the WT parasites used in the former study. These differences may alter the dynamics of immune responses. Nevertheless, the results agree with the notion that type I IFNs play distinct roles under different malaria conditions.

Overall, the available data indicate that type I IFNs play disparate roles in malaria infection depending on the timing and amount of production, relative levels of IFN-α, IFN-β and perhaps other isomers, and compositions of cellular and cytokine milieu during the progression of infection, and parasite strains. A recent review provides a comprehensive and up-to-date account on the role and contrasting effects of type I IFNs in malaria (180). Type I IFNs are pleiotropic cytokines and as such, they induce a wide range of effects on innate and adaptive immune cells during various pathogenic infections, contributing to either protection against infection or pathogenesis (181–183). These differential effects are likely dependent on the levels of type I IFNs; low levels at early stages of infection mediate cell-mediated immunity, but high levels cause immunosuppression (182). Thus, it is not surprising that type I IFNs produced during malaria infection also induce a wide range of cellular effects involving a complex interplay between various cell types.

In addition to producing type I IFNs, DCs produce a wide range of pro-inflammatory cytokines, including TNF-α, IL-12, and IL-6, and chemokines, such as CXCL1, CXCL2, CCL2, CCL5, CXCL9, and CXCL10 in response to malaria parasites, and play crucial roles in malaria immunity and pathogenesis (34, 38, 108, 184–186). Type I IFNs prime DCs for efficient cytokine and chemokine production and activate NK, NKT, γδ T, and T cells to induce IFN-γ and other inflammatory responses (182). IL-12 produced by DCs activates NK cells to induce the production of IFN-γ, which promotes Th1 and effector T cell responses (187). The augmented production of IFN-γ contributes to an efficient parasitemia control by priming Mφs and neutrophils for increased phagocytic activity and thus parasite clearance (188, 189). IFN-γ also contributes to cerebral and other severe malaria clinical conditions under certain situations, such as parasite sequestering in vital organs (187, 188, 190). Chemokines, on the other hand, promote the recruitment of immune cells to mount effective cell-mediated anti-parasitic effects (191). However, these responses also contribute to severe pathology (37, 192, 193). By and large, the initial innate immune responses are mainly aimed at controlling parasite growth by potentiating antiparasitic cell-mediated immunity. However, since pro-inflammatory responses contribute to pathogenesis (14, 33–38), as infection progresses, the function of DCs switches from pro-inflammatory and Th1-inducing to anti-inflammatory and Th2-inducing phenotypes (39, 40, 194). Eventually, balanced pro- and anti-inflammatory and Th1/Th2 responses prevent pathogenesis and promote protective humoral immunity to malaria (39–42); imbalanced responses contribute to pathogenesis. Thus, innate immune responses contribute to either protective immunity or pathogenesis in a context dependent manner.

TLR-MYD88 Signaling Prominently Contributes to Protective Immunity and Pathogenesis

DNA and RNA play prominent roles in mouse malaria immunity and pathogenesis; under certain conditions TLR2 and TLR4 also play important roles. As such, studies in various mouse models have demonstrated that TLR9, TLR7, TLR4, and TLR2 play important roles in malaria immunity and cerebral, placental and other severe malaria pathology (43, 44, 81, 102, 103, 106–108, 124, 128, 174, 195–201). Gene polymorphism studies in endemic areas that assessed the role of TLRs in malaria have linked TLR9, TLR4, and TLR2 to either susceptibility or resistance to malaria (202–214). While the involvement of TLR9, TLR4, and TLR2 in malaria immunity/pathology is evident from studies in both mouse malaria models and in humans (43, 106, 195, 197, 198, 200–203, 205, 210), thus far none of the studies in endemic areas have revealed the association of TLR7 with human malaria immunity/pathology. It is unclear whether studies in endemic areas that have assessed association of TLRs have investigated the role of TLR7 in malaria susceptibility/resistance. However, since *P. falciparum* RNA appears to have very low immunostimulatory activity, it is possible that TLR7 has either minor or no role in human malaria. Accumulated evidence indicates that TLRs prominently contribute malaria immunity and pathology, whereas other signaling mechanisms such as inflammasome and adherence-mediated signaling may play minor roles (172, 215).

The contribution of pro-inflammatory responses produced by the innate immune system to protective immunity and pathogenesis is primarily context dependent. In non-cytoadherent parasites, such as *P. yoelii* 17XNL strain and *P. chabaudi* that exhibit slow growth rates during the initial phase of infection, pro-inflammatory responses are protective by facilitating anti-parasitic effector function and cell-mediated immunity. In contrast, in the case of cytoadherent parasites,

such as *P. berghei* ANKA that sequesters in brain, lungs, liver and adipose tissues, and *P. berghei* NK65 that sequesters in lungs and liver, pro-inflammatory responses are pathogenic by promoting cytotoxicity in effector cells, which cause organ damage. In this regard, the observations made in mouse models parallel those of vivax and falciparum malaria in humans. In *P. falciparum* infection, strong pro-inflammatory responses contribute to an effective control of parasitemia which otherwise results in fulminant infection (41, 42). However, despite this beneficial effect, pro-inflammatory responses promote effector cell function, which contributes to organ damage and severe illnesses. In contrast, in the case of *P. vivax*, which is relatively slow growing and does not cytoadhere except at low levels in lungs and the placenta (125), despite causing a number of clinical conditions, fatal illnesses are relatively rare compared to *P. falciparum*.

CONCLUDING REMARKS

Malaria continues to be a major global health problem. In many endemic areas, parasites are becoming increasingly resistant to the currently widely used artemisinin-based combination drugs, which have been very effective in treating infection. Therefore, new drugs or other treatment strategies are urgently needed. Mass vaccination is the best strategy to prevent malaria. However, despite huge efforts during the past decades in many laboratories around the world, obtaining an efficacious vaccine suitable for mass vaccination remains challenging. It is thought that better understanding of molecular and cellular processes involved in the development of protective immunity and those that contribute to severe pathogenesis will be useful in designing an effective vaccine. During the past decades, a significant progress has been made in identifying the parasite factors and host receptors involved in the activation of the innate immune system and the associated cell signaling pathways. Significant progress has also been made in understanding how cellular activation and immune responses initiated upon parasite-host interactions influence subsequent development of antiparasitic immunity and contribute to severe pathogenesis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Dendritic Cell Responses and Function in Malaria

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Malaria remains a serious threat to global health. Sustained malaria control and, eventually, eradication will only be achieved with a broadly effective malaria vaccine. Yet a fundamental lack of knowledge about how antimalarial immunity is acquired has hindered vaccine development efforts to date. Understanding how malaria-causing parasites modulate the host immune system, specifically dendritic cells (DCs), key initiators of adaptive and vaccine antigen-based immune responses, is vital for effective vaccine design. This review comprehensively summarizes how exposure to *Plasmodium* spp. impacts human DC function *in vivo* and *in vitro*. We have highlighted the heterogeneity of the data observed in these studies, compared and critiqued the models used to generate our current understanding of DC function in malaria, and examined the mechanisms by which *Plasmodium* spp. mediate these effects. This review highlights potential research directions which could lead to improved efficacy of existing vaccines, and outlines novel targets for next-generation vaccine strategies to target malaria.

Keywords: dendritic cells, malaria, *Plasmodium falciparum*, *Plasmodium vivax*, vaccines

INTRODUCTION: MALARIA

Malaria remains one of the greatest challenges to public health in the developing world. It is caused by infection with the *Plasmodium* species of Apicomplexans, which have a complex life cycle spanning multiple organ sites (**Figure 1**), facilitated by multiple morphologically and antigenically distinct life stages, and expression of multiple antigens (1–5).

The *Plasmodium* life cycle bridges two hosts: mosquitoes, where sexual replication occurs, and humans, where the parasite undergoes asexual replication. The latter begins when an infected mosquito injects sporozoite-stage parasites from mosquito salivary glands into the skin (**Figure 1**). A small fraction of sporozoites will travel to the liver, where the sporozoite will traverse hepatic tissue until it locates a suitable hepatocyte. The subsequent exoerythrocytic form will release merozoites into the bloodstream upon rupture (6). *Plasmodium vivax* can also enter a dormant liver stage known as the hypnozoite, which can mature and produce merozoites weeks to years after the initial infection (7, 8). Despite being only 1 μm in size, the merozoite expresses a range of parasite proteins that ligate host red blood cell (RBC) ligands to drive invasion. After invasion the merozoite forms a parasitophorous vacuole in host cells, where it begins to mature into a trophozoite (9).

From 18 to 32 h post-invasion, the trophozoite increases DNA replication and metabolic activity. The mid-trophozoite stage exports various parasite proteins, including those crucial to host pathology, such as the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (10). At 34 h post-invasion, the parasite becomes a

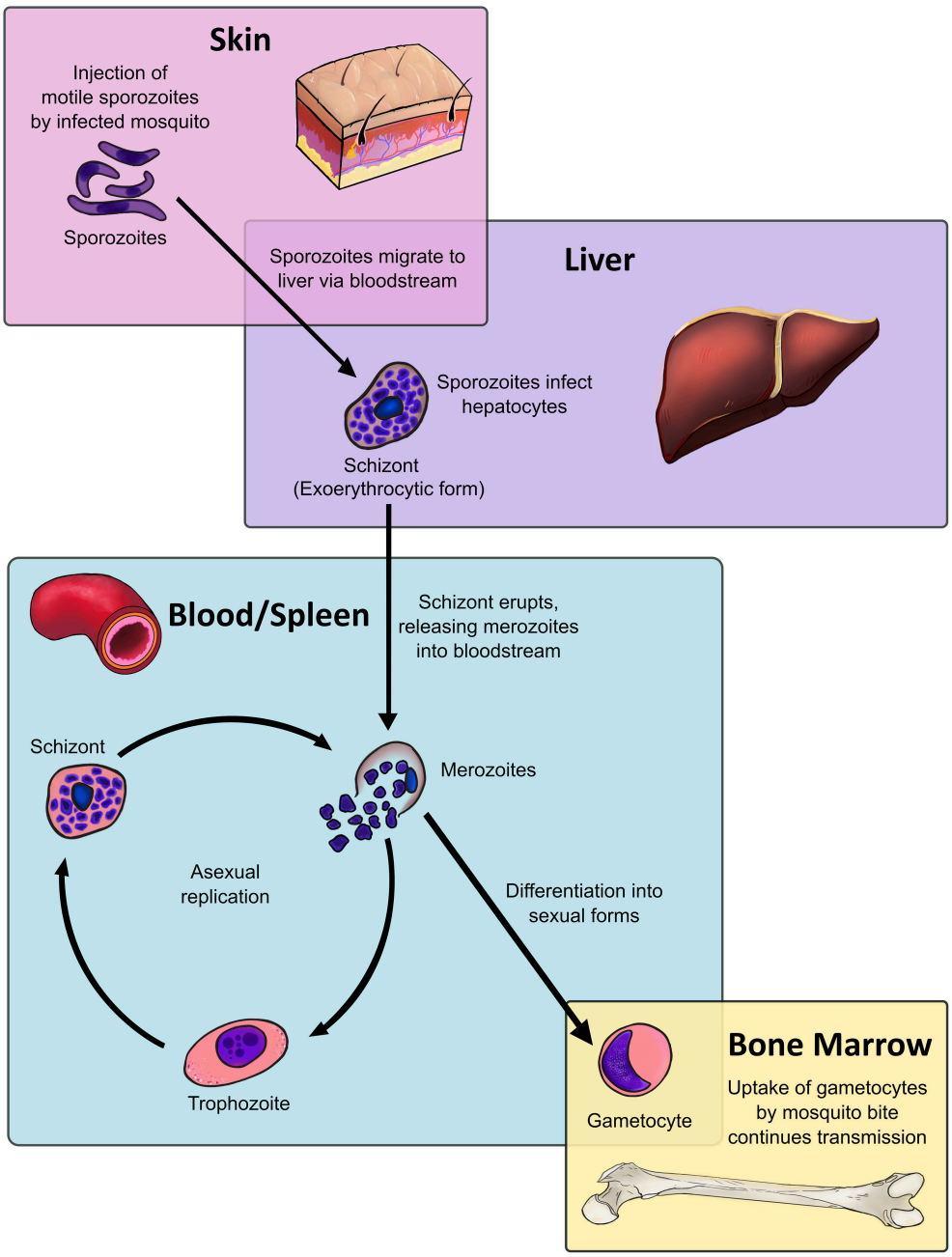

FIGURE 1 | Dendritic cells, located throughout the body at various stages of maturity, interact with all stages of the malaria parasite life cycle within the human host. The *Plasmodium* life cycle encompasses multiple life stages across a range of tissues. The asexual life cycle in the human host begins when mosquitoes inject sporozoites, the highly motile infectious life stage, into the host's skin. The sporozoite migrates to the liver, where it traverses multiple host cells before entering into an exoerythrocytic form. The exoerythrocytic form matures into a multinucleate schizont, which releases merozoites into the bloodstream upon lysis. Merozoites infect host red blood cells and mature into intraerythrocytic life stages known as trophozoites, which are highly metabolically active. After DNA replication the trophozoite will become a blood-stage schizont, which will lyse and release daughter merozoites into the bloodstream, resuming the process. Instead of becoming trophozoites, a fraction of merozoites will instead differentiate into sexual stages known as gametocytes, which sequester in the bone marrow. Only at the end of their maturation process do gametocytes re-enter the bloodstream, where they are taken up by mosquito bite to commence sexual replication in the mosquito host and continue the cycle.

multinucleate, segmented stage known as the schizont. After 48 h of intracellular maturation and replication, the schizont ruptures, destroying the erythrocyte and releasing parasite metabolites, waste products, and between 16 to 32 daughter merozoites are released into the bloodstream (9), where the cycle will begin afresh.

After 7–15 days in circulation, a small proportion of *P. falciparum* trophozoites will commit to sexual replication, where the process of schizogony is replaced by the formation of sexual stages known as gametocytes (11, 12). Generation of *P. vivax* gametocytes is much faster, with gametocytes being detectable in circulation from 3 days post-infection (13, 14). Gametocytes undergo five maturation stages: stages I-IV preferentially sequester in the bone marrow (BM) and spleen (15–17) while stage V gametocytes re-enter the circulation, where they can be taken up by the bite of infected mosquitoes (18).

The effect of each malaria life stage on host immune function is not well understood, nor are the broader underlying mechanisms of antimalarial immunity. It is frequently observed that individuals living in highly endemic regions develop clinical immunity against symptomatic disease, but generally do not develop sterilizing immunity that completely protects against infection. Antibodies are a crucial component of naturally acquired clinical immunity, as passive transfer of immunoglobulins from malaria immune to non-immune individuals is sufficient to reduce parasitaemia and resolve symptoms (19). Furthermore, clinical immunity appears in most cases to be relatively short-lived and broadly declines in the absence of boosting [reviewed in (20)]. An improved understanding of antimalarial immunity will enable development of future vaccines which can accelerate acquisition of clinical immunity, or better yet, induce sterile immunity.

Malaria Vaccines

The most advanced malaria vaccine candidate to date is RTS,S, which targets the circumsporozoite protein (CSP) of *P. falciparum*. RTS,S has shown modest efficacy in Phase III clinical trials, with 29 and 36% efficacy in young infants and young children, respectively over 3–4 years, with a booster dose given at 20 months (21). The sub-optimal efficacy of RTS,S and its failure to elicit protective immunity in many recipients is poorly understood (21–23). To elucidate the immunological responses that future malaria vaccines should aim to induce or improve upon, it is vital to understand how different parasite life stages modulate the host immune system. This review focuses specifically on the interactions between malaria parasites and dendritic cells (DCs), sentinel antigen presenting cells of the immune system that are crucial for generating effective immune responses and immunological memory.

Dendritic Cells

DCs function as a crucial bridge between innate and adaptive immunity. In a healthy individual, DCs constitute only 1% of all peripheral blood mononuclear cells (PBMC) (24–26), yet they exert potent regulatory effects on both the innate and adaptive immune system (**Figure 2**). Upon encountering foreign antigens in the presence of pathogen associated molecular patterns (PAMPs), DCs undergo a process of maturation and migrate to the spleen and draining lymph nodes where they interact with pathogen-specific T cells. In addition to presenting antigen via major histocompatibility complex (MHC) surface molecules, DCs express co-stimulatory molecules required for naïve T cell proliferation and differentiation into effector cells, including CD40, CD80 (B7-1), and CD86 (B7-2). Through secretion of cytokines and chemokines, DCs recruit other immune cells and influence the nature of the adaptive T and B cell response, ultimately leading to clearance of infected cells and extracellular pathogens (**Figure 2**). Crucially, DCs are present at all clinically relevant sites for the development of *Plasmodium* life stages, namely the skin, blood, bone marrow, spleen, and liver (**Figure 1**).

Based on the expression of CD11c and CD123, human DCs can be broadly classified into plasmacytoid DCs (pDC; $Lin^-HLA\text{-}DR^+CD11c^-CD123^+$) and conventional DCs (cDC; $Lin^-HLA\text{-}DR^+CD11c^+CD123^-$) populations. The pDCs are the body's major producers of the anti-viral interferon (IFN)-α, though they constitute only 0.35% of PBMCs (25, 26). These cells are crucial in antiviral responses. The cDCs specialize in priming and presenting antigen to T cells (27), and constitute 0.6% of PBMCs (25, 26). Using the blood dendritic cell antigen (BDCA) markers, it is possible to further differentiate cDC populations into cDC1 ($BDCA\text{-}3^+/CD141^+$) and cDC2 ($BDCA\text{-}1^+/CD1c^+$) subsets, while pDCs express BDCA-2 (CD303) and BDCA-4 (CD304) (28–30).

Given the central role of DCs in sensing infection and orchestrating immune responses, it is not surprising that many pathogens have evolved immune evasion strategies which specifically target DCs in order to interfere with innate and adaptive immune responses (31–34). Thus, understanding how DCs initiate and maintain effective immune responses against malaria parasites, whilst minimizing detrimental and life-threatening immunopathology, is imperative for vaccine development.

AT THE MEETING POINTS: SITES OF DC AND *PLASMODIUM* SPP. INTERACTION

Interactions between DCs and *Plasmodium* parasites occur at every stage of the parasite life cycle within the human host: skin (35), liver (36), and most importantly within the blood and spleen (37), where the majority of host pathology occurs. Recent studies have also revealed that the bone marrow (BM) compartment is a major tissue reservoir for gametocyte development and proliferation of malaria parasites (38–41). Tissue-resident DCs in each of these sites have the potential to endocytose parasite components and initiate the development of specific adaptive immune responses to *Plasmodium* infection. Importantly, DCs in these tissues exist in different maturation states and thus vary in their ability to influence adaptive and innate immune responses and induce inflammatory responses. Within the liver, DCs are thought to induce tolerogenic responses to prevent induction of harmful immunopathology (42, 43), whilst in spleen, DCs propagate strong immune responses, and blood DCs have an intermediate phenotype with a lower capacity for inducing inflammation compared to their splenic counterparts (44).

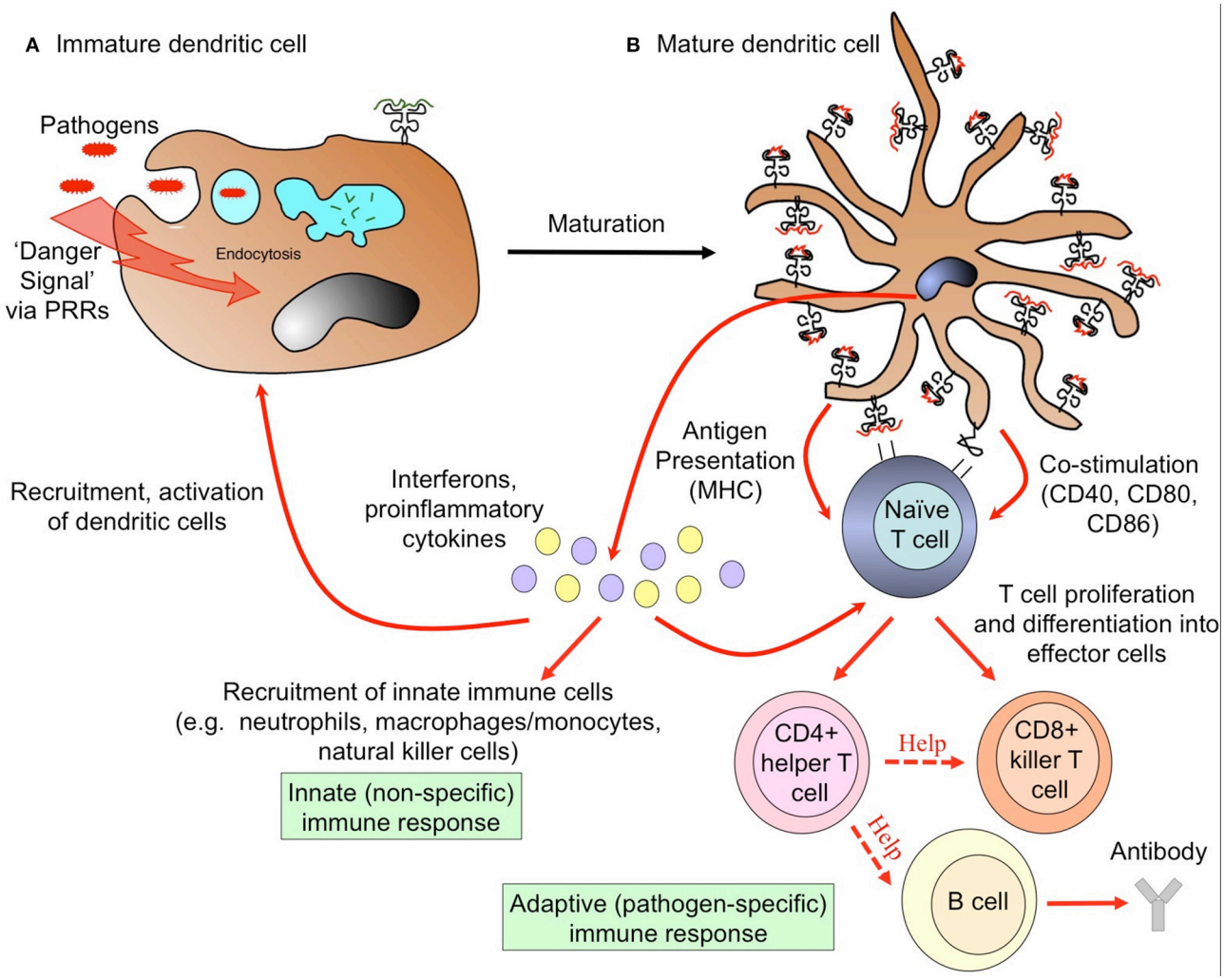

FIGURE 2 | Dendritic cells link innate and adaptive arms of the immune system. **(A)** Uptake of pathogens and recognition of pathogen-associated "danger signals" by pattern recognition receptors (PRRs) triggers dramatic morphological and functional changes in DCs, termed maturation. These changes involve the formation of dendrites, down-regulation of antigen uptake, and redistribution of major histocompatibility complex (MHC) molecules from intracellular endocytic compartments to the cell surface. **(B)** Mature DCs migrate to draining lymph nodes and present information about the invading pathogen in the form of processed peptides loaded onto MHC molecules to naïve T cells. Upregulation of MHC and co-stimulation molecules enables activated DCs to initiate adaptive T and B cell immune responses, the nature of which are determined by the cytokine milieu. This initiates the cascade to an adaptive immune response, leading to clearance of infected cells, and extracellular pathogens. Activated mature DCs also secrete interferons and proinflammatory cytokines that recruit circulating innate immune cells to provide rapid defense against infection.

Skin and Liver DC Interactions With Sporozoites: Lessons From Murine Models

The skin is the site of first contact between DCs and *Plasmodium* spp. Studies in mice have demonstrated that sporozoites remain in the skin for up to 60 min prior to entering the circulation, after which they lose motility (45). Remarkably, up to 50% of sporozoites become trapped in the dermis, while 30% of those that succeed in entering the circulation enter lymphatic rather than blood vessels (45). Thus, the majority of sporozoites fail to reach the bloodstream and are instead phagocytosed by DCs in the skin-draining lymph nodes, which prime protective $CD4^+$ (46–48) and $CD8^+$ T cell responses (49, 50). It is likely that a substantial proportion of immunity to sporozoite stages arises predominantly in response to these "failed" sporozoites.

Interestingly, there is some evidence that sporozoites which arrest within the liver may promote induction of limited liver-stage immunity. A murine study demonstrated that apoptosing hepatocytes infected by irradiated sporozoites triggered recruitment of circulating blood DCs to the liver (51). These DCs phagocytosed apoptotic hepatocytes and migrated to lymph nodes, where they induced protective IFN-γ-producing $CD8^+$ T cell responses (50).

Importantly, in the above study, infiltrating DCs from the cutaneous lymph nodes initiated immune responses, not

liver-resident DCs (50). In humans (52) and mice (53), tissue-resident liver DCs are reportedly less mature than blood DCs, as they are poor at antigen processing and express only low levels of costimulatory markers. While liver DCs in humans are capable of inducing allogeneic T cell responses, they are less effective at this than their blood counterparts, and therefore promote a T cell phenotype that is less responsive to subsequent stimulation (52, 54). When considered in conjunction with their high capacity for IL-10 secretion (52), the liver DC phenotype may be one that promotes a more tolerogenic environment, favorable to sporozoite survival. This could partly explain why sterile immunity rarely occurs in response to natural infection, with tolerogenic liver-resident DCs acting to suppress inflammatory responses which would induce protection. Studies using mouse models with humanized livers have shown promise for investigating *Plasmodium* spp. skin-to-liver transfer (55, 56). In combination with FMS-like tyrosine kinase 3 ligand (Flt3-L)-treated cord blood engrafted humanized mice, which produce large quantities of human DCs similar to those seen in blood (57), combined liver-immune system humanized mice could be a useful avenue to investigate DC involvement in liver-stage immunity.

The Bone Marrow As a Reservoir for Gametocytes

A similar phenomenon of immune tolerance may occur in the BM, which emerging evidence suggests is a privileged developmental niche for the transmission stages of *Plasmodium*. Autopsy studies have indicated that both *P. vivax* and *P. falciparum* (15, 58–61) gametocytes sequester in the BM, the latter of which is supported by the presence of a *Pf*EMP1 type capable of binding BM endothelium (62). Poor immune responses to parasites in this milieu may be due to tolerogenic potential of the BM microenvironment. There is very little data on BM DCs. One non-human primate study indicated that BM-derived $CD123^+HLA\text{-}DR^+$ pDCs had a decreased capacity to express co-stimulatory molecules in response to pathogens relative to blood DCs (63), while $CD11c^+$ BM cells in a murine study had a similar capacity for T cell stimulation relative to their blood and spleen counterparts (64). However, it is not clear whether the $CD11c^+$ population in the latter study was comprised solely of DCs.

No studies to date have examined how DCs in the BM respond to sequestered parasites, although one murine study has reported that pDCs, present in the BM at frequencies 20 times higher than in the blood or spleen, are the major producers of IFN-α during *P. yoelii 17X YM* infection (65). If the BM is indeed a reservoir for infection, as is suggested by recent primate studies (41), studying whether BM DCs are capable of initiating antimalarial immune responses will be important for achieving elimination.

Blood and Spleen DC Interactions With Malaria Blood-Stages

Blood-stage parasitaemia provides multiple opportunities for blood and splenic DCs to interact with parasites. The parasite spends the majority of the asexual blood-stage cycle within the host RBC. While *P. vivax* exclusively infects reticulocytes, which express surface MHC and can therefore be cleared by $CD8^+$ T cells (66), *P. falciparum* also infects mature RBCs, which do not express surface MHC molecules, thus enabling host immune evasion. Despite this, the blood-stage is an antigenically rich phase of the *Plasmodium* life cycle [reviewed in (67, 68)], affecting a large proportion of host cells and triggering potent inflammatory immune responses that cause most of the symptoms of malaria. Maturation of parasitized RBCs (pRBCs) culminates in lysis of the host RBC, releasing merozoites into the circulation. Merozoites that fail to invade a new RBC will remain in the circulation where they are directly phagocytosed (69) or circulate to the spleen for clearance. The *Pf*EMP1 molecule, which is expressed on the pRBC surface, may play a dual role in this life stage. While it is a prime target for antibodies in naturally acquired immunity (70), one report suggests it may also modulate immune function via binding to CD36 on APCs, including DCs (71). Furthermore, *Pf*EMP1-mediated sequestration in the periphery is long held to be a parasite adaptation aimed at avoiding splenic clearance (72).

DCs play a vital role in initiating and regulating adaptive immunity to blood-stage malaria (73–75). However, there is strong evidence that *Plasmodium* parasites modulate DC maturation and function to interfere with the development of protective immune responses. Data from mouse models indicate that blood-stage infection suppresses both existing and developing liver-stage immunity by inhibiting DC activation (76), and inhibits DCs from responding to subsequently encountered pathogens (77–79). Importantly, murine studies suggest that DCs also play a role in the induction of immune-mediated pathology, including the life-threatening syndrome of cerebral malaria (80, 81). Thus, it is of vital importance that we understand the factors governing the ability of DCs to alter the balance between protection and pathology.

DCs, Malaria, and Unanswered Questions

The majority of DC-*Plasmodium* interactions in humans have been studied in two ways: (1) studying peripheral blood DCs from currently or previously infected individuals, or (2) measuring DC responses to parasite stimuli *in vitro*. In the first method, DCs were isolated from the blood of individuals who were naturally or experimentally infected with malaria. The surface phenotype and function of these DCs was compared to uninfected controls, either the same individuals prior to or post-infection, or a matched control group (82–102). In the second method, DCs from malaria-naïve individuals were stimulated with *Plasmodium* products to assess the resulting phenotype. The majority of reports which used the latter generated DCs from monocytes *in vitro* using GM-CSF and IL-4 (71, 103–106), while a minority reported responses from *bona fide* DCs from blood (83, 85, 107, 108).

As such, there is limited knowledge about how naïve DC subsets resident in different human tissues and blood respond to *Plasmodium*, and what factors influence this response. This knowledge is vitally important for designing vaccine strategies which specifically enhance the ability of DCs to induce protective responses while limiting induction

of immunopathology. Understanding how naïve DC function is altered by *Plasmodium* exposure will provide insight into how DCs are affected in infected individuals, and therefore what vaccine strategies will be required to overcome this altered phenotype.

PERIPHERAL BLOOD DC RESPONSES TO NATURAL OR EXPERIMENTAL *PLASMODIUM* INFECTION

A total of 24 *ex vivo* studies to date have examined how natural or experimental exposure to *Plasmodium* spp. affects the activation phenotype and function of human peripheral blood DCs, in both acute infection and after prior exposure (summarized in **Table 1**). The following sections analyse these studies in detail, according to species infection.

DC Phenotypes and Responses During *P. falciparum* Infection

Plasmodium falciparum is responsible for a high burden of morbidity and mortality in pregnant women and children, and can cause severe and fatal disease outcomes including cerebral malaria, miscarriage, and multiple organ failure (110). Infected persons typically present to hospital when blood-stage infection becomes symptomatic, which can occur nine to 30 days after the initial infection (111). Classifying malaria cases as mild/uncomplicated vs. severe is based on specific clinical features, including but not limited to coma, haemoglobinuria, vital organ dysfunction, or respiratory distress (110). The majority of *ex vivo* studies have been carried out in settings of high *P. falciparum* transmission, focusing on the phenotype and function of DCs in high-risk groups including children and pregnant women (**Table 1**).

DCs and *P. Falciparum* in Children in High-Transmission Settings

In a DC study comparing infected children to non-infected controls in a holoendemic setting, Kenyan children hospitalized with mild vs. severe malaria exhibited decreased HLA-DR expression on DCs and reduced DC numbers in circulating blood, regardless of disease severity (82). A subsequent study which followed children during malaria and after treatment showed that malaria specifically decreased HLA-DR expression on cDC but not pDC subsets, and reduced the ability of DCs to induce allogeneic T cell proliferation in mixed leucocyte reactions (MLR) (96). Furthermore, infection correlated to an increase in absolute numbers of circulating BDCA-3$^+$ cDC1s. Importantly, these effects of *P. falciparum* on DC phenotype and function were still observed 14 days after hospital discharge and curative treatment (96), suggesting that malaria-induced immunosuppression can persist for some time after parasite clearance.

A subsequent study was conducted in Mali, another holoendemic setting, where DC function was compared between infected and non-infected children from the Fulani and Dogon ethnic groups. DCs from children aged 2–10 years displayed reduced HLA-DR expression after malaria exposure (100). Infection was also associated with increased proportions of circulating BDCA-2$^+$ pDC and BDCA-3$^+$ cDC1 populations, with reduced CD86 expression in the former (100). In this study genetic differences were proposed to play a role in clinical outcomes of *P. falciparum* infection due to differences in cytokine production between the 2 ethnic groups, with PBMCs from Dogon children displaying significantly impaired cytokine production, correlating with more severe fever and higher parasitaemia (100). These responses could be attributed in part to reduced DC function, including a reduction of pDC-derived IFN-α production in response to TLR9 ligands.

More recently, Guermonprez et al. reported that children with malaria, regardless of disease severity, had an increased frequency of the BDCA-3$^+$ cDC1 population (102). This correlated with increased serum concentrations of the DC growth factor Flt3-L that preferentially increases pDC and cDC1 *in vivo* (112, 113). During malaria, Flt3-L is produced by mast cells in response to uric acid metabolism by *Plasmodium* parasites (102).

Together, these studies suggest that malaria in children in high-transmission settings negatively impacts DC activation marker expression and modulates DC function. The low activation status of peripheral DCs may be due to sequestration of activated DCs in affected tissues. Moreover, an increased number of circulating BDCA-3$^+$ cDC1s appears to be a common feature of malaria in this setting. Urban et al. also showed that DC dysfunction persisted after the resolution of malaria (96), leaving these individuals vulnerable to co-infections. The apparent contradiction between reduced DC numbers in the first study (82) and elevated numbers of BDCA-3$^+$ cDC1s in the second study (96) is likely due to more sophisticated gating strategies in the latter, enabling discrimination of individual DC subsets (96), rather than classifying all HLA-DR$^+$ cells as DCs (82). Rigorous and well-defined flow cytometry gating strategies that use an appropriate combination of antibodies to DC subset-specific surface markers are imperative for DC research and may help to resolve some of the apparent discrepancies in the literature.

DCs and *P. Falciparum* in Pregnancy in High-Transmission Settings

Four studies evaluating changes in DC populations in infection during pregnancy have yielded conflicting results. Two studies, one from Gabon (94) and one from Benin and Tanzania (101), observed that overall DC numbers were decreased in pregnant women infected with *P. falciparum* compared to uninfected matched pregnant controls, while a study from Senegal (97) reported a decrease in the pDC population only relative to non-pregnant uninfected controls. Another study from Benin (109) did not observe any difference in DC numbers between infected and non-infected pregnant women. Changes in surface activation marker expression varied across studies (**Table 1**).

Again, different gating strategies may underlie some of the differences observed between these studies. Simply gating on CD123$^+$ or CD11c$^+$ populations may run a risk of false positives if isolation and lineage staining is not extensive enough. Use of cord blood (94, 97) or placenta-derived (97) DCs may also

TABLE 1 | *In vivo* exposure to *Plasmodium* species modulates human DC responses.

References	Cohort demographic	Transmission intensity (country)	DC subset gating strategy (phenotype)	Changes in surface molecule expression	Serum /plasma cytokines	Other effects
P. falciparum						
Urban et al. (82)	Children	Holoendemic (Kenya)	HLA-DR$^+$, CD83$^+$	Decreased: HLA-DR No change: CD83	Increased: TNF-α IL-10	Decreased DC numbers
Pichyangkul et al. (83)	Hospitalized adults	Mesoendemic (Thailand)	cDC (HLA-DR$^+$CD11c$^+$) pDC (HLA-DR$^+$CD123$^+$)		Increased: IFN-α	Reduced numbers of circulating pDC
Breitling et al. (94)	Pregnant women	Holoendemic (Gabon)	cDC (BDCA1$^+$) pDC (BDCA2$^+$)	No change: HLA-DR*		Decrease in overall DC numbers
Urban et al. (96)	Children	Holoendemic (Kenya)	cDC (CD11c$^+$BDCA1$^+$, CD11c$^+$BDCA3$^+$) pDC (CD123$^+$BDCA2$^+$)	Decreased: HLA-DR (cDC)	Increased: TNF-α IL-10 IL-12	Elevated number of BDCA-3$^+$ cDC1 in circulating blood during and after malaria infection Decreased cDC ability to induce allogeneic T cell proliferation
Diallo et al. (97)	Pregnant women	Hypoendemic (Senegal)	cDC (CD11c$^+$CD123^{lo}) pDC (CD11c$^-$CD123^{hi}) Less differentiated DC (CD11c$^-$CD123 ^{lo})	No change: CD83* Decreased: HLA-DR*	Increased: TNF-α IFN-γ IL-10	Women who have had malaria have higher percentages of less differentiated DC Decreased circulating pDC in infected pregnant women
Loharungsikul et al. (98)	Hospitalized adults	Mesoendemic (Thailand)	cDC (BDCA1$^+$, BDCA3$^+$) pDC (BDCA2$^+$)	Increased: TLR2 (cDC) Decreased: TLR9 (pDC) No change: TLR4 (cDC)		Decreased fraction of TLR2$^+$ cDC in peripheral blood during infection
Fievet et al. (109)	Pregnant women	Mesoendemic (Benin)	cDC (BDCA1$^+$, BDCA3$^+$) pDC (BDCA2$^+$)	Increased: HLA-DR (BDCA1, BDCA2) No change: CD86* HLA-DR (BDCA3)	No change: TNF-α IFN-γ IL-10 IL-12 IL-6 MIP-1α	Increased percentage of HLA-DR positive BDCA2$^+$ cells during infection Decreased absolute number of all DCs from women with ≥3 pregnancies Increased pDC number in women with ≥3 pregnancies
Gonçalves et al. (99)	Clinic admission	Hypo- to mesoendemic (Brazil)	cDC (HLA-DR$^+$CD11c$^+$) pDC (HLA-DR$^+$CD123$^+$)	No change: CD86*	Increased: IFN-γ TNF-α IL-10	Decreased cDC number Decreased total DC number

(Continued)

TABLE 1 | Continued

References	Cohort demographic	Transmission intensity (country)	DC subset gating strategy (phenotype)	Changes in surface molecule expression	Serum /plasma cytokines	Other effects
Arama et al. (100)	Children	Mesoendemic (Mali)	cDC ($HLA\text{-}DR^+BDCA1^+$, $HLA\text{-}DR^+BDCA3^+$, $HLA\text{-}DR^+CD16^+$) pDC ($HLA\text{-}DR^+BDCA2^+$)	Decreased: HLA-DR* CD86 (pDC)	Decreased: IFN-γ	Increased $BDCA\text{-}2^+$ pDC and $BDCA\text{-}3^+$ cDC1 populations in peripheral blood Impairment of TLR signaling in DC during malaria results in more severe clinical symptoms
Ibitokou et al. (101)	Pregnant women	Mesoendemic (Benin) Holoendemic (Tanzania)	cDC ($HLA\text{-}DR^+BDCA1^+$) pDC ($HLA\text{-}DR^+BDCA2^+$)	Decreased: HLA-DR (cDC) Increased: CD86 (pDC)		Decreased pDC and cDC fraction in peripheral blood
Guermonprez et al. (102)	Children	Holoendemic (Kenya)	cDC ($CD11c^+BDCA1^+$, $CD11c^+BDCA3^+$) pDC ($CD123^+BDCA2^+$)		Increased: Flt3L	Increased $BDCA\text{-}3^+$ cDC1 fraction in peripheral blood Increased $CD8^+$ T cell activation in peripheral blood
Pinzon-Charry et al. (84)	Infected adults	Holoendemic (Papua)	cDC ($HLA\text{-}DR^+CD11c^+$) pDC ($HLA\text{-}DR^+CD123^+$) iDC ($HLA\text{-}DR^+CD123^-CD11c^-$)	Decreased: HLA-DR CD83 CD86	Increased: TNF-α IFN-γ IL-2 IL-4 IL-6 IL-10	Decreased pDC and cDC fractions Increased fraction of immature DC Increased DC apoptosis Decreased ability to induce T cell proliferation Decreased antigen uptake
Restimulation of DCs from naturally *P. falciparum*-infected individuals[†]						
Fievet et al. (109)	Pregnant women	Mesoendemic (Benin)	cDC ($BDCA1^+$, $BDCA3^+$) pDC ($BDCA2^+$)	No change: HLA-DR (cDC)	Increased: IFN-γ TNF-α IL-10 No change: IFN-α IL-6 IL-12 MIP-1α	Increased production of proinflammatory cytokines by women ≤25 years independent of gravidity
Götz et al. (85)	Adults	Holoendemic (Mali)	cDC ($HLA\text{-}DR^+BDCA1^+$, $HLA\text{-}DR^+BDCA3^+$, $HLA\text{-}DR^+CD16^+$)	Increased: HLA-DR CD86 No change: CD40 CD80	Increased: CCL2 CXCL9 CXCL10 No change: IL-1β IL-6 IL-10 TNF-α CCL5	

(Continued)

TABLE 1 | Continued

References	Cohort demographic	Transmission intensity (country)	DC subset gating strategy (phenotype)	Changes in surface molecule expression	Serum /plasma cytokines	Other effects
P. vivax						
Jangpatarapongsa et al. (86)	Hospitalized adults	Mesoendemic (Thailand)	cDC ($HLA\text{-}DR^+CD11c^+$) pDC ($HLA\text{-}DR^+CD123^+$)		Increased: IL-10	Decreased fraction of cDC and pDC during infection Increased numbers of $FOXP3^+$ T_{REG} during infection
Gonçalves et al. (99)	Clinic admission	Hypo- to mesoendemic (Brazil)	cDC ($HLA\text{-}DR^+CD11c^+$) pDC ($HLA\text{-}DR^+CD123^+$)	*Decreased*: CD86*	Increased: TNF-α IL-10	Increased pDC fraction Decreased cDC number Decreased total DC number
Pinzon-Charry et al. (84)	Infected adults	Holoendemic (Papua)	cDC ($HLA\text{-}DR^+CD11c^+$) pDC ($HLA\text{-}DR^+CD123^+$) iDC ($HLA\text{-}DR^+CD123^-CD11c^-$)	Decreased: HLA-DR CD83 CD86	Increased: TNF-α IFN-γ IL-2 IL-4 IL-6 IL-10	Decreased pDC and cDC fractions Increased fraction of immature DC Increased DC apoptosis Decreased T cell proliferation Decreased antigen uptake
***P. falciparum* and *P. vivax* coinfection**						
Gonçalves et al. (99)	Clinic admission	Hypo- to mesoendemic (Brazil)	cDC ($HLA\text{-}DR^+CD11c^+$) pDC ($HLA\text{-}DR^+CD123^+$)	No change: CD86*	Increased: TNF-α IL-10	Decreased cDC fraction Increased pDC fraction
Kho et al. (87)	Children, adults, hospitalized children, and adults	Holoendemic (Papua)	cDC ($HLA\text{-}DR^+BDCA1^+$, $HLA\text{-}DR^+BDCA3^+$) pDC ($HLA\text{-}DR^+BDCA2^+$)	Increased: HLA-DR* Decreased: HLA-DR (cDC)		Increased pDC fraction during asymptomatic *P. vivax* but not *P. falciparum* infection Decrease in $BDCA\text{-}1^+$ cDC2 fraction during asymptomatic infection with either species or during uncomplicated malaria
Kho et al. (88)	Adults	Holoendemic (Papua)	cDC ($HLA\text{-}DR^+BDCA1^+$, $HLA\text{-}DR^+BDCA3^+$) pDC ($HLA\text{-}DR^+BDCA2^+$)	Decreased: HLA-DR		Decreased numbers of circulating pDC and $BDCA\text{-}1^+$ cDC2 during symptomatic infections but not during subpatent infections Reduced $CD4^+$ T cell proportion Decreased proportion of activated and resting T_{REG}
Controlled human malaria infection with *P. falciparum*						
Woodberry et al. (89)	Healthy adult males	N/A (CHMI)	cDC ($HLA\text{-}DR^+CD11c^+$) pDC ($HLA\text{-}DR^+CD123^+$)	Decreased: HLA-DR (pDC)	No change: TNF-α IL-6 IL-10 IL-12	Increased DC apoptosis Decreased overall DC numbers Decreased phagocytic activity

(Continued)

TABLE 1 | Continued

References	Cohort demographic	Transmission intensity (country)	DC subset gating strategy (phenotype)	Changes in surface molecule expression	Serum /plasma cytokines	Other effects
Teirlinck et al. (90)	Healthy adults	N/A (CHMI)	cDC (HLA-DR$^+$BDCA1$^+$, HLA-DR$^+$BDCA3$^+$) pDC (HLA-DR+BDCA2$^+$) CD16$^+$ DC (HLA-DR$^+$CD16$^+$ CD14$^-$)	Increased: HLA-DR (CD16$^+$, pDC) CD86 (CD16$^+$) CD16 (CD16$^+$, BDCA-1$^+$, pDC) CD1c (CD16$^+$, BDCA-1$^+$, pDC) No change: HLA-DR (cDC) CD86 (cDC, pDC)		Increased expression of BDCA-1 and CD16 on all subsets except BDCA-3$^+$ cDC1 Increased CD1c/BDCA-1 and CD16 expression on monocytes after treatment
Loughland et al. (91)	Healthy adults	N/A (CHMI)	cDC (HLA-DR$^+$CD11c$^+$ BDCA-1$^+$)	Decreased: HLA-DR CD86	Increased: TNF-α No change: IL-12	Increased DC apoptosis Decreased antigen uptake Decreased overall DC numbers
Loughland et al. (92)	Healthy adults	N/A (CHMI)	pDC (HLA-DR$^+$CD11c$^-$CD123$^+$)	Decreased: HLA-DR No change: HLA Class I	No change: TNF-α Increased: IFN-α	Reduced circulating pDC at and 24 h after peak parasitaemia Increased caspase-3 expression in pDCs Increased expression of *NLRC5*, *C14orf119* and *TSG101* Decreased expression of *DMBT1*, *AREGB*, *RNF139*, *CRYM*, and *BAG3*
Loughland et al. (93)	Healthy adults	N/A (CHMI)	CD16$^+$ DC (HLA-DR$^+$CD14$^-$CD11c$^+$BDCA-1$^-$CD16$^+$CD86$^+$)	Decreased: CD16 Increased: HLA-DR CD86	Increased: TNF-α IL-10	Increased proportion of CD16$^+$ DCs among all CD11c$^+$ DCs Non-parasite-specific loss of CD16 when DCs are in culture
Controlled human malaria infection with *P. vivax*						
Woodberry et al. (95)	Healthy adults	N/A (CHMI)	cDC (HLA-DR$^+$CD11c$^+$ BDCA1$^+$, HLA-DR$^+$CD11c$^+$ BDCA3$^+$) pDC (HLA-DR$^+$CD123$^+$) CD16$^+$ DC (HLA-DR$^+$CD11c$^+$ CD16$^+$)	Decreased: HLA-DR (cDC) CD123* No change: HLA-DR (pDC)	Increased: IFN-γ	Reduced circulating pDC, BDCA-1$^+$ and BDCA-3$^+$ DC Increased caspase-3 expression in pDC, CD16$^+$, and BDCA-1$^+$ cDC Increased numbers of activated T_{REG} during infection Increased indoleamine 2,3-dioxygenase metabolism drives T_{REG} differentiation

*Transmission intensity, reported intensity of transmission in the catchment area at the time of sample collection; DC subset gating strategy, subsets and markers used to define the subsets which were examined in the study; changes in surface molecule expression, change in activation marker expression after exposure to Plasmodium, relative to healthy controls; serum/plasma cytokines, cytokines assayed for in serum or plasma, change measured relative to healthy controls (no stimulation/no exposure); other effects, other observed changes in cell populations, numbers, or function. *All DC subsets affected. †Surface molecule expression and cytokine secretion for DCs in these studies were measured in supernatants of purified DC cultures after restimulation with pRBCs (85) or TLR ligands (109), as opposed to direct measurement of DC phenotypes from whole blood.*

contribute to phenotypic differences between these DCs and peripheral blood DCs, due to the unique microenvironments of these pregnancy-associated tissues. Gravidity can also be an important contributing factor. Since primigravid women are at the highest risk of severe inflammatory disease [reviewed in (114)], the proportion of women in their first pregnancy should always be accounted for in immunological studies. Inclusion of pregnant non-infected controls is also imperative to determine whether pregnancy itself is a confounding factor affecting DC function during malaria.

Function of DCs From Naturally Exposed Individuals

Three studies of adults with symptomatic malaria carried out in Thailand (98), Brazil (99), and Papua (84) provide insights into how *P. falciparum* immunity develops in lower-transmission settings. Within the Thailand cohort, activation marker expression was not assessed, but circulating numbers of pDCs were significantly reduced in both mild and severe malaria compared to healthy controls. IFN-α levels in the serum increased (83), but it was not established whether this directly correlated with pDC function. The percentage of immature HLA-DR$^+$CD11c$^-$CD123$^-$ cells in circulation increased, while the fractions of circulating CD11c$^+$ cDCs and CD123$^+$ pDCs were decreased. DCs from infected participants were apoptotic (upregulated the apoptotic marker Annexin-V) and were defective at antigen uptake and induction of naïve T cell proliferation in allogeneic T cell activation assays (84). All three cohorts were recruited via clinical admissions, which self-selects for individuals with lower pre-existing immunity and perhaps a more naïve phenotype.

In short, it appears that while impairment is more pronounced in high-transmission settings due to frequent re-infection and higher overall parasite burden, downregulation of DC function is a common feature of malaria. Considering that malaria induces potent inflammation, this DC phenotype may therefore be comparable to what is seen in other inflammatory diseases such as bacterial sepsis (115), HIV (116), or HCV (117). In these patients it is also common to observe reductions in circulating DC numbers (115, 116) and reduced HLA-DR (117) or CD86 (115, 117) expression. Persistent systemic inflammation may therefore explain this reduction in DC function in naturally malaria-infected persons. Again, more rigorous classification of cDC1, cDC2, and pDCs may clarify some of the discrepancies amongst different reports.

Stimulation of DCs From Naturally Exposed Individuals

In a study examining DC responses to TLR stimulation after natural *P. falciparum* infection, DCs from naturally exposed pregnant women in Benin were collected from cord blood (109). Whole PBMC cultures were stimulated with TLR4 ligand LPS, TLR3 ligand polyinosinic:polycytidylic acid (polyI:C), or TLR9 ligand CpG-A ODN to stimulate BDCA-1$^+$ cDC2, BDCA-3$^+$ cDC1, or pDCs, respectively, due to the high expression of each TLR on these specific DC subsets (118). Synthetic hemozoin prepared from haemin chloride was also used for DC stimulation. There was no difference in HLA-DR expression between infected and non-infected women upon stimulation with either TLR ligands or hemozoin. PBMCs from infected women produced more TNF-α and IL-10 in response to CpG-A stimulation, more IFN-γ in response to polyI:C, and more TNF-α in response to hemozoin relative to non-infected women (109).

Only one study to date has stimulated DCs from naturally exposed individuals using pRBCs (85). DCs were purified from the blood of adults from a highly endemic region in Mali at the end of the transmission season and DC activation was compared to that in naïve controls. All exposed individuals were PCR-negative for infection at the time of enrolment (85). When stimulated with pRBCs at a ratio of 3 pRBCs per DC, DCs from these individuals upregulated expression of HLA-DR and CD86 and expressed CCL2, CXCL9, and CXCL10, but did not produce any IL-1β, IL-6, IL-10, or TNF-α (85). In Section 4, this review outlines how a lack of cytokine secretion is commonly observed in *in vitro* studies of *bona fide* DC, and therefore should not necessarily be considered a sign of DC suppression. However, it is interesting that when DCs isolated from malaria-exposed individuals were stimulated with pRBCs following cessation of high malaria transmission (85), DCs could express an activatory surface phenotype in response to stimulation. Thus, it may be that sustained reductions in transmission allow restoration of DC function.

TLR Modulation in DCs by *P. falciparum*

Only one study to date has investigated the ability of *P. falciparum* to modulate TLR expression on DCs as a potential mechanism of immune suppression (98). In this study, individuals with severe or mild *P. falciparum* infection exhibited increased TLR2 expression on cDCs but decreased TLR9 expression on pDCs, with no observable change in TLR4 expression (98) compared to healthy controls. The severity of infection did not impact these changes in TLR expression. Moreover, the fraction of TLR2$^+$ DCs in the periphery decreased during infection (98). TLR2, TLR4, and TLR9 have all been implicated in sensing of *Plasmodium*-derived "danger signals." Namely, TLR2 and TLR4 recognize glycophospholipid (GPI) anchors for merozoite surface proteins (119), and TLR9 detects *Plasmodium* DNA (120). As this is the only study to assess TLR expression profiles during *Plasmodium* infection, it is unclear whether this effect is a common feature of malaria. Nevertheless, it suggests that even low-level *Plasmodium* infections can modulate host responses by downregulating the signals required for APC activation.

The Effects of Natural *P. vivax* Infection on DC Phenotype and Function

Plasmodium vivax is the second major malaria pathogen. It inhabits a broader geographical range than *P. falciparum,* posing a risk to more than 3.2 billion individuals worldwide (121). Its pathogenic potential is enhanced by its ability to become a latent hypnozoite in the liver (7), but as it exclusively infects reticulocytes (122), it is difficult to maintain in culture and remains relatively understudied. Immunity to *P. vivax* has primarily been studied in symptomatic persons who present to healthcare. As the geographical ranges of *P. vivax* and *P. falciparum* transmission overlap, it is often difficult to exclude

the immunological impact of prior *P. falciparum* exposure. Nonetheless, it is possible to describe the acute effects of *P. vivax* single-species infection, even though an individual's infection history may be unclear, if diagnosis is sufficiently rigorous. The gold standard for species-specific diagnosis is PCR. However, in resource-poor settings rapid diagnostic tests are typically used.

Due to the paucity of studies from *P. vivax*-exposed individuals it is difficult to conclude the effects of *P. vivax* malaria on DC function. DC numbers decreased during infection, both as a fraction (84, 86) and as total numbers (99). In the latter study, the pDC fraction was increased while cDC numbers decreased (99). Another study observed a decrease in both pDC and cDC fractions, as well as increased DC apoptosis (84). *Plasmodium vivax* malaria has also been reported to down-regulate CD86 expression on DCs (84, 99).

The Effect of Mixed *Plasmodium* Infections on DC Function

Phenotypic analyses of peripheral blood DC from individuals co-infected with two *Plasmodium* spp. support similar reductions in overall DC numbers as seen in individuals experiencing single infections (87, 88, 99). However, it is not yet known whether this correlates to impairments in DC function. A study from Gonçalves et al. in a mesoendemic area of Brazil found that asymptomatic individuals infected with both *P. falciparum* and *P. vivax* had decreased circulating cDCs but increased circulating pDCs (99). Studies in a holoendemic region of Papua found that pDC fractions increased during asymptomatic *P. vivax* but not *P. falciparum* infection, with pDC and BDCA-1$^+$ cDC2 fractions decreasing during acute infection with either species (87, 88). No changes were observed in the BDCA-3$^+$ cDC1 fraction in children or adults during acute or asymptomatic infection with either species (87, 88), in contrast to the findings in African cohorts (96, 100, 102). HLA-DR expression on DCs was increased during asymptomatic *P. vivax* infection (87), but decreased during acute mixed or single-species infections (87, 88).

It is interesting that HLA-DR expression on DCs was positively correlated with parasitaemia in children with asymptomatic *P. vivax* infection, but negatively correlated with parasitaemia in adults with asymptomatic *P. falciparum* infection (87). Thus, it may be that the two major pathogenic *Plasmodium* species polarize the immune system in different ways. This data also suggests fundamental differences in how childrens' and adults' DCs respond to *Plasmodium* exposure—an important factor to keep in mind considering the at-risk populations for either species.

Insights From Controlled Malaria Infection Models

Controlled Human Malaria Infection With *P. falciparum*

The development of a controlled human malaria infection model (CHMI) has produced valuable insights into antimalarial immunity. In one CHMI model which has been used to study DC in malaria, healthy volunteers who are typically malaria-naïve were inoculated with an ultra-low (<180) or low (1,800) dose of *P. falciparum* pRBCs thawed from a pre-prepared biobank. Atovaquone/proguanil or artemether/lumefantrine treatment was administered 6 days post-infection (ultra-low-dose group) or when parasitaemia reached 1,000 parasites per mL (low-dose group). Despite the low parasite biomass of the inoculum in the low-dose group, an estimated 20 times lower than the number of merozoites released from an infected hepatocyte after sporozoite replication (123), DC numbers were significantly decreased in the low-dose group due to increased DC apoptosis (89). Intriguingly, infection-induced apoptosis appeared to be exclusive to HLA-DR$^+$ cells, including DCs. Furthermore, the decrease in DC numbers coincided with the peak of symptomatic malaria, and while cDC numbers recovered to pre-infection levels after drug treatment, pDC numbers remained at 47% of baseline 60 h post-cure (89). HLA-DR expression on pDCs was also impaired. Importantly, DCs from the low-dose group displayed impaired phagocytosis, which persisted for 36 h after drug cure. In contrast, the ultra-low-dose group experienced no symptoms and no DC impairment (89). This study suggests that a certain parasite biomass is required for functional impairment of DCs. However, since the ultra-low-dose group were treated prior to development of symptoms, it is unclear whether an ultra-low dose is sufficient to induce immunity that can control sub-symptomatic parasitaemia, or whether immune impairment would have eventuated if parasitaemia had been allowed to develop.

Function of pDCs and BDCA-1$^+$ cDC2s during CHMI

A second controlled infection study from Loughland et al. utilized a similar low- (1800 pRBCs) and ultra-low (150 pRBCs) dose to more closely study BDCA-1$^+$ cDC2 activation (91) and pDC function (92) after controlled infection. Unlike the prior study, patients were treated upon reaching a parasitaemia of 1000 pRBCs per mL, regardless of initial parasite inoculum. Importantly, both groups experienced a decrease in HLA-DR expression on BDCA-1$^+$ cDC2s that coincided with peak parasitaemia but also persisted 24 h after drug treatment (91). However, only the high-dose group exhibited decreased DC numbers, increased DC apoptosis, and reduced phagocytic capacity relative to baseline (91, 92). A positive association was also observed between phagocytic activity and HLA-DR expression at peak parasitaemia (91).

The ability of DCs to respond to TLR stimulation after exposure to malaria was also evaluated in these studies (91) by restimulating DCs taken from participants during peak parasitaemia. Interestingly, the BDCA-1$^+$ cDC2 from individuals in the high-dose group were impaired in their capacity to upregulate HLA-DR and CD86 in response to stimulation with TLR1/2, TLR4, and TLR7 ligands or whole pRBCs. This impairment was DC-specific, as monocytes' capacity for activation marker expression was unaltered by malaria exposure (91). In contrast, pDCs restimulated with TLR7 and TLR9 ligands upregulated expression of HLA-DR, CD123, and IFN-α, and upregulated CD86 in response to TLR7 stimulation (92). The cDC1 subset was not examined in these studies. These results were similar to TLR stimulations of cord blood

DCs from pregnant women, where CpG-A stimulation of pDCs showed enhancement of cytokine production in infected individuals (109), though caution must be taken when comparing naïve CHMI participants to naturally-exposed pregnant women in Benin.

Together, these studies suggest that a single infection is sufficient to impair cDC function, while pDC function is more resilient. As discussed further on, this highlights a need to further study pDC function during malaria and the potential role of this subset in immunopathology.

CD16$^+$ DC function in CHMI

The CD16$^+$ DC subset's status as a steady-state DC rather than a monocyte subset that acquires DC-like characteristics during inflammation remains unclear (27, 124, 125). Improved strategies for distinguishing "true" CD16$^+$ DCs from CD16$^+$CD14$^-$ monocytes have not yet been established, although a recent single-cell RNAseq study highlighted a population of BDCA-1$^-$BDCA-3$^-$CD16$^+$ cDCs that is transcriptomically distinct from monocytes (126). However, two studies have examined the role of CD16$^+$ "DCs" in malaria, both in CHMI. Both studies observed that relative to pre-CHMI levels HLA-DR and CD86 expression in these DCs increased after curative treatment (90, 93) and 24 h prior to peak parasitaemia (93). At peak parasitaemia CD16$^+$ DCs had an increased ability to spontaneously produce TNF-α, IL-10, and IL-12. CD16$^+$ DCs collected at peak parasitaemia and restimulated with pRBCs expressed higher levels of IL-10 relative to baseline (93). When restimulated with TLR1/2 or TLR4 ligands, these CD16$^+$ DCs produced high levels of TNF-α and moderate amounts of IL-10 and IL-12. When restimulated with TLR7 ligands, the CD16$^+$ DCs produced TNF-α only (93). While caution must be taken in ascribing *bona fide* DC status to the CD16$^+$ DCs, these studies indicate that these cells are activated during infection and in the highly inflammatory environment post-treatment. Their high production of both TNF-α and IL-10, which may aid in killing or suppression of DCs, respectively, suggest that they could be major contributors to DC modulation, including that seen many days post-treatment and clearance of infection (96).

CHMI With *P. vivax*

Due to the technical difficulty of maintaining *P. vivax* in continuous culture, to date only one CHMI has been published using *P. vivax* (95). In this study, peripheral DC numbers were significantly reduced during acute infection relative to baseline, though this was concurrent with an overall reduction in circulating PBMC (95). All subsets (BDCA-3$^+$ cDC1s, BDCA-1$^+$ cDC2s, pDCs, and CD16$^+$ cDCs) upregulated caspase-3 during acute infection and after treatment, suggesting that the reductions in DC numbers in the periphery could also be due to increased apoptosis. Overall, DC impairment by *P. vivax* CHMI was largely similar to what was observed with *P. falciparum* (89, 91); HLA-DR expression on BDCA-1$^+$ cDC was reduced during acute infection and 24 h after treatment (95).

Ex vivo DCs in *Plasmodium* Infection: What Do We Know?

In summary, *Plasmodium* infection can result in reduced DC numbers in the periphery, both as an absolute number (89, 91, 94, 99) and as a proportion of total leucocytes (82, 97, 101), reportedly due to increased DC apoptosis (84, 89, 91). DC capacity for phagocytosing antigen is also decreased (89, 91), which correlates with DC activation (127), yet their ability to induce T cell proliferation in allogeneic T cell stimulation assays is impaired (84, 89, 96). HLA-DR expression is generally decreased (87–89, 91, 92, 95–97, 100, 101), with some variability between DC subsets (**Table 1**). It is not clear whether the reduction in HLA-DR is due to an increase in new immature DCs in the circulation, or direct downregulation by parasites. There is little consensus regarding other markers: reports on CD83 (84, 97) and CD86 expression are contradictory, though CD86 tends to be elevated on pDCs and decreased on DCs as a total population (83, 84, 91, 100, 101).

It is also unclear whether the decrease in the number of circulating DCs is due to cell death, as suggested by the upregulation of caspase-3 (89, 91, 95) or annexin V (84), or due to increased migration to lymphoid tissues. Decreased DC numbers in both natural and experimental infection, however, coincided with increased serum levels of IL-10 (82, 84, 86, 96, 97, 99) and TNF-α (82, 84, 91, 96, 97, 99), indicating a potential cytokine-mediated mechanism of DC loss. One subset in particular defied this trend: proportions of BDCA-3$^+$ cDC1s were increased during *P. falciparum* infection (96, 100, 102), and remained elevated for some time after acute infection (96). The BDCA-3$^+$ cDC1 subset is associated with the initiation of CD8$^+$ killer T cell responses and the secretion of IL-12 (128). It is likely that increases in serum Flt3-L lead to increased numbers of these DC in the periphery during infection, but these circulating DC do not appear to be capable of inducing functional responses. Further complicating the matter, the BDCA-3$^+$ cDC1 subset is not elevated in single or mixed infections from Papua, where transmission intensity is comparable (87, 88).

Overall, the different methods and markers that have been used to study DCs in this variety of settings makes it difficult to clearly define universal parameters of DC loss of function. It is possible that the DC downregulation described in these studies is a feedback loop promoting regulatory mechanisms in the face of severe malaria-induced inflammation, and that DC downregulation in malaria is not necessarily detrimental to host survival. However, the presence of functional DCs is required for effective vaccine responses, and it is still not clear how malaria-induced DC downregulation affects survival to other pathogens. There is an overall need to understand how these DC phenotypes correlate to clinical outcomes, or at minimum, how malaria directly affects DC function. It will be important to clarify whether DC downregulation during natural infection translates to suppression, namely loss of generalized immune function against non-malaria pathogens or inflammatory stimuli.

In light of this, *in vitro* studies of DC function are vital for three purposes: (1) clarifying the phenotype of DC suppression, (2) determining precisely how malaria modulates DC function,

and (3) identifying whether this is through direct interaction with DCs or indirectly through soluble mediators, including cytokines such as TNF-α.

DEFINING THE INTERACTIONS BETWEEN DCS AND *PLASMODIUM* SPP. *IN VITRO*

To date, relatively few studies have investigated direct interactions between *Plasmodium* spp. and human DCs *in vitro*. The majority of these studies have examined the responses of human monocyte-derived DCs (moDCs), since they can be easily generated in large numbers from $CD14^+$ PBMCs or BM monocytes by co-culture with GM-CSF ± IL-4 (129, 130). MoDC are themselves heterogeneous and contain cells with a cDC-like phenotype with high expression of MHC class I and II, BDCA-1, CD40, CD80, and CD11c (129), and macrophage-like cells (131). Transcriptomic analysis indicates that moDC are highly distinct from blood $CD16^+$, cDC2 ($BDCA\text{-}1^+$), and cDC1 ($BDCA\text{-}3^+$) cDC subsets and therefore do not accurately represent the diversity of DC populations or their functions *in vivo* (124). Other recent findings indicate that monocyte-derived inflammatory DCs in humans are more similar to macrophages than to *bona fide* DCs [reviewed in (27, 125)]. Thus, moDCs may not be a representative model for investigating *bona fide* human DC responses. These caveats must be considered when interpreting the data from *in vitro* studies (summarized in **Table 2**).

MoDCs and Intracellular *P. falciparum* Blood-Stage Parasites

Initially, *P. falciparum* pRBCs were thought to suppress moDC function *in vitro* (103) as, when co-cultured with moDCs at a concentration of 100 parasites per DC, they impaired moDC activation via contact-dependent CD36-mediated mechanisms (103). In this study, DCs co-incubated with CD36-binding parasite lines displayed decreased expression of co-stimulatory markers CD40, CD54/ICAM-1, CD80, CD83, and CD86 in response to LPS stimulation, and had a low capacity for inducing allogeneic T cell proliferation (103). Co-incubation with non-CD36 binding parasite lines did not induce the same inhibition. However, a subsequent study found that a high ratio of pRBCs to DCs (100:1) inhibited LPS-induced DC maturation, cytokine production, and allogeneic T cell stimulation regardless of whether the parasite strain had a CD36-binding phenotype, and low doses of parasite (10:1) induced modest DC maturation and autologous T cell proliferation (104). This inhibition of LPS-induced DC maturation with high doses of pRBCs was co-incident with high levels of DC death *in vitro* (104).

Another study reported that a ratio of 10 pRBCs per moDC did not trigger upregulation of HLA-DR, CD83, or CCR7 on moDCs (132), contradicting the findings of Elliott et al. (104). However, the 100:1 ratio induced secretion of IL-1β, IL-6, IL-10, TNF-α, and upregulation of the pro-migratory chemokine receptor CXCR4 (132). Another report indicated that even at a ratio of 25 pRBCs per moDC, moDCs upregulated HLA-DR, CD40, CD80, and CD83 and secreted significantly higher levels of TNF-α, IL-6, and IL-10 (105). At higher pRBC-to-DC ratios, there was a corresponding increase in DC death (105).

Addition of CD40L to pRBC-stimulated moDCs enhanced HLA-DR and CD80 expression while CD86 expression was greatly reduced relative to CD40L alone (105). Secretion of TNF-α, IL-12, and IL-6 was also enhanced, while IL-10 secretion was unchanged relative to CD40L alone (105). In another study, exposure to schizont lysate triggered moDCs to upregulate CD86 but not CD80 or HLA-DR (106). These lysate-stimulated moDCs were capable of inducing allogeneic T cell differentiation into T_H1 and regulatory T cells (T_{REG}), both of which secreted high levels of IFN-γ. T_{REG} induced in this fashion also secreted high levels of IL-10 and TGFβ. Pre-incubating moDCs with parasite lysate did not affect their ability to undergo LPS-driven maturation (106). Lastly, moDCs stimulated with whole schizonts did not upregulate HLA-DR, CD80, or CD86, nor did they express cytokines or chemokines (**Table 2**) (85).

One explanation proposed by Elliott et al. (104) for the conflicting literature on the effect of pRBCs on moDC activation is that high ratios of pRBCs suppress DC function, while low ratios activate DCs (104), though in a recent study moDCs were not activated by stimulation with 3 pRBCs per DC (85). Alternately, variations in methodology are likely to contribute to some of the differences observed: different parasite strains and co-culture periods were used across all studies (**Table 2**). Moreover, the heterogeneity of moDC preparations can vary widely amongst different laboratories. Schizont lysate is also not a proxy for pRBCs as the lytic process produces a mixture of parasite membrane proteins, metabolites, and merozoites (107). The matter is further complicated by the multiple ways of defining "inhibition": whether pRBCs truly block DC activation in response to an external stimulus, and which stimuli in particular are susceptible to this manner of inhibition. Alternatively, it must be clarified whether pRBCs induce higher levels of DC death.

In summary, while a dose-dependent relationship between pRBC dose and moDC inhibition is suggested, this relationship must be substantiated by further studies examining the individual roles that different parasite stimuli, strains, and methodological factors have on the final DC phenotype, preferably focusing on *bona fide* DCs in future studies. A more rigorous definition of moDCs and how "activation" and "inhibition" are defined in these cells, particularly given how different groups have used different activatory cytokine stimulation methods to drive DC-like cells to begin with, will be imperative to resolve existing conflicts.

Monocyte-Derived DCs and Other *Plasmodium* Life Stages

Human DC responses to other *Plasmodium* life stages have been poorly investigated. Only one study has investigated moDC responses to *P. falciparum* merozoites (105). In this study, co-incubation with merozoites resulted in moDC secretion of TNF-α, IL-16, and large amounts of IL-10, despite no changes in costimulatory marker expression (105). Co-incubating merozoites with moDCs in the presence of

TABLE 2 | DC responses after *in vitro* exposure to *P. falciparum*.

References	DC type	Parasite strains	Stimulus type	Post-stimulation phenotype	Cytokines detected	Other effects	Proposed mechanism of action
Monocyte-derived DCs*							
Urban et al. (103)	Monocyte-derived	ITO/A4 ITO/C24 MC T9/96	Whole trophozoite	Decreased: CD40 ICAM-1 CD80 CD83 CD86		Suppressed DC ability to induce T cell activation DC suppression mediated by CD36 binding	CD36 ligation by *PfEMP1*
Urban et al, (71)	Monocyte-derived	ITO/C24 MC	Whole trophozoite	Decreased: HLA-DR CD83	Increased: IL-10 TNF-α Decreased: IFN-γ IL-4 IL-12 No change: TGF-β	Suppressed DC ability to induce T cell activation Co-incubation with CD40L does not restore DC function Apoptotic, but not necrotic cells have similar suppressive function	CD36 ligation by *PfEMP1*
Elliott et al, (104)	Monocyte-derived	ItG E8B CS2 3D7/upsCSBP1-KO	Whole trophozoite	Decreased: HLA-DR CD40 CD80 CD83 CD86	Decreased: IL-10 IL-12	pRBC lysate does not suppress LPS-mediated DC activation and can activate DCs High-dose pRBCs induce DC apoptosis Both CSA- and CD36-binding pRBCs inhibit LPS-mediated T cell proliferation	Parasite-to-DC ratio; *PfEMP1*-independent
Mukherjee and Chauhan, (105)	Monocyte-derived	3D7	Whole trophozoite	Increased: HLA-DR CD80 Decreased: CD86	Increased: TNF-α IL-6 IL-12 No change: IL-10	Stimulate $CD4^+$ T cells to produce IFN-γ, IL-5, and IL-10	Phosphorylation of p38MAPK
			Free merozoite	Decreased: HLA-DR CD80 CD83 No change: CD86	Increased: IL-10 Decreased: IL-12 No change: TNF-α IL-6	Decreased phosphorylation of p38MAPK Stimulate $CD4^+$ T cells to produce IL-10, low amounts of IFN-γ	Modulation of IL-10/IL-12 secretion via altering ERK1/2 signaling
Clemente et al. (106)	Monocyte-derived	3D7	Schizont lysate	Increased: CD86 No change: HLA-DR CD80	No change: IL-10 IL-12	Malaria-exposed DCs induce naive T cell differentiation into T_H1 and T_{REG} subsets	

(Continued)

TABLE 2 | Continued

References	DC type	Parasite strains	Stimulus type	Post-stimulation phenotype	Cytokines detected	Other effects	Proposed mechanism of action
Götz et al. (85)	Monocyte-derived	3D7	Intact schizont	No change: HLA-DR CD80 CD86	No change: CCL2 CCL5 CXCL9 CXCL10 IL-1β IL-6 IL-10 TNFα		
***Bona fide* DCs**[†]							
Pichyangkul et al. (83)	Blood pDC (HLA-DR$^+$CD123$^+$)	TM267R GR MRU LA PH	Intact schizont Schizont lysate	Increased: CD86 CCR7 No change: CD40	Increased: IFN-α No change: TNF-α	Schizont-stimulated pDCs can induce γδ T-cell proliferation and IFN-γ production	
Wu et al. (107)	Blood cDC (HLA-DR$^+$BDCA1$^+$) Blood pDC (HLA-DR$^+$BDCA2$^+$)	3D7	Merozoite lysate		Increased: TNF-α IL-12p40 IFN-α	pDC-cDC cross-signaling is required for cytokine secretion by DCs	Cell-to-cell contact between DC subsets and other immune cells
Gowda et al. (108)	Blood cDC (HLA-DR$^+$BDCA1$^+$) Blood pDC (HLA-DR$^+$BDCA4$^+$)	3D7	Intact trophozoite	Increased: CD36	Increased: TNF-α No change: IL-12p40	DCs internalize CD36-binding pRBCs more efficiently	CD36 ligation by *PfEMP1*
Götz et al. (85)	Blood cDC (HLA-DR$^+$BDCA1$^+$) Blood pDC (HLA-DR$^+$BDCA4$^+$)	3D7	Intact schizont Schizont lysate	Increased: HLA-DR CD40 CD80 CD86	Increased: CCL2 CXCL9 CXCL10 IFN-α No change: CCL5 IL-1β IL-6 IL-10 TNF-α	Low ratios of pRBCs do not suppress DC activation by LPS pRBC-primed DCs induce T_H1-like responses pDC-cDC cross-signaling is required for production of IFN-α, CXCL9 and CXCL10	NFκB- and PPARγ-independent

DC type, how DCs used in this study were derived; Parasite strain(s), P. falciparum laboratory strains used for stimulation; Stimulus type, parasite life stage used in this study and whether parasites were purified or processed. [*] *Monocyte-derived DCs, induced by co-incubating adherent precursors from whole blood with IL-4 and GM-CSF for 24 h (129).* [†] *Bona fide DCs, DCs which have full DC function after purification from donor tissues, without requiring any cytokine maturation.*

CD40L induced high CD86 expression but no increase in other costimulatory surface markers (105). CD40L also induced merozoite-stimulated moDCs to produce high levels of IL-10 (105).

Likewise, only a single study to date has assessed moDC responses to *P. vivax* sporozoites (36). Prior to co-culture, moDCs were matured with TNF-α and LPS and primed, or not, with sporozoite extract. Primed moDC were more efficient than their unprimed counterparts at eliciting IFN-γ secretion and autologous T cell proliferation in DC-T cell co-cultures, and $CD8^+$ T cells stimulated by primed moDCs had greater cytotoxic effector activity against infected HC04 hepatocyte lines (36). It is not yet known how DCs respond to other liver-stage parasites such as hypnozoites, exo-erythrocytic forms, or sexual-stage gametocytes.

Interactions Between *Bona fide* Human DCs and *P. falciparum*

Due to the technical challenges in obtaining large numbers of viable *bona fide* human DCs from peripheral blood, relatively few studies have investigated direct interactions between *ex vivo* blood DCs from healthy donors and *P. falciparum* merozoites or pRBCs (**Table 2**). To date, studies have focused on BDCA-1^+ cDC2 and pDC populations. None have examined the BDCA-3^+ cDC1 subset, likely owing to the rarity of this population. Both merozoites and pRBCs have been shown to induce blood DCs to upregulate CD40, CD80, and CD86 (83, 85), and to secrete IFN-α (83, 85, 107), indicating that *P. falciparum* is capable of activating naïve DCs. Merozoites also triggered production of IL-12p40 and TNF-α (107). Additionally, a ratio of 3 pRBCs per DC resulted in upregulation of HLA-DR and increased expression of the chemokines CCL2, CXCL9, and CXCL10 (IP-10) (85), but did not trigger production of IL-1β, IL-6, IL-10, or TNF-α. Contrary to findings in moDCs, pRBCs did not suppress cytokine responses to LPS in *bona fide* DC, although this may be attributable to the lower pRBC-to-DC ratio used in this study (85). While the authors did not assess whether high doses of pRBCs modulated the ability of *bona fide* DCs to prime naïve T cells, as was shown for moDCs, *bona fide* DCs exposed to low doses of pRBCs were fully functional in their antigen presenting ability, inducing naive T cell proliferation and polarization toward an IFN-γ-producing T_H1 phenotype (85). This does suggest, congruent with moDC studies (104, 106) and some CHMI studies (89, 91), that single, low-parasitaemia blood-stage infections of 10 pRBCs per DC or fewer, equivalent to 200 pRBCs/μL, may induce beneficial DC activation.

It is likely that cross-talk between different DC subsets plays an important role in immune responses to *P. falciparum*. Two studies that have examined this process indicate that DC cross-talk is required for production of TNF-α, IL-12p40 (107), IFN-α, CXCL9, and CXCL10 (also known as IP-10) (85) in response to pRBCs. In the context of antimalarial responses, DC activation appears to be contact-mediated and independent of IFN-α, although partially mediated by the TLR9 pathway (85, 107), expressed by just the pDC subset of human DCs. While cDCs alone are sufficient for inducing T cell activation to pRBCs, the presence of pDCs affects the ability of activated T cells to proliferate and produce cytokines (85). When a mixed culture of pDC and cDC was used in pRBC-primed autologous T cell stimulations, T cells trended toward reduced proliferation and production of IL-10, TNF-α, IFN-γ, and IL-5, but increased IL-2 secretion (85). It is possible that since the overall number of DCs for T cell stimulations was kept constant, reduced T cell activation was a consequence of the reduced proportion of cDCs.

These data highlight a need for future studies to investigate not only the individual roles of *bona fide* DC subsets in immunity to malaria, but also to consider the complexity of the immune response and the influence of cell-to-cell interactions. This should be reflected in the establishment of better *in vitro* models and cell-based systems that more realistically mimic the dynamic interactions and cell behaviors that occur over the course of an immune response *in vivo*.

DC Interactions With Parasite by-Products

The cycle of parasite reproduction is fuelled by a range of host nutrients, not least of which is intraerythrocytic hemoglobin. Hemoglobin breakdown causes accumulation of toxic heme, which the parasite neutralizes by aggregating heme crystals into hemozoin (133). Hemozoin has been proposed to have both suppressive and activatory effects on DCs.

Initial studies reported that purified hemozoin induced CD1a, CD80, and CD83 upregulation and IL-12 secretion from moDCs, whereas monomeric heme and synthetic hemozoin (β-hematin) did not (134). However, these results were contradicted by a subsequent study demonstrating impaired upregulation of HLA-DR, CD40, CD80, CD83, ICAM-1, and CD1a in moDCs pre-incubated with *P. falciparum* hemozoin (135). These conflicting results may be due to the use of different hemozoin sources. Depending on the method of purification, parasite hemozoin can be contaminated with parasite proteins, nucleic acids, and other by-products that can activate alternate pathways. It is possible for even purified hemozoin to adhere to environmental contaminants after purification (133). The altered activity of hemozoin on treatment with phospholipase D (135) and DNAse (136) indicate that contamination with nucleic acids or other parasite metabolites is a likely explanation for the observed variations, particularly since hemozoin has been shown to be a carrier for *Plasmodium* DNA (136).

Murine models have been vital in establishing how hemozoin-malaria DNA complexes activate DCs. Murine Flt3-ligand-induced DCs (137) stimulated with hemozoin chelated to *P. falciparum* DNA secreted high levels of RANTES, IL-12, and TNF-α. In this system, hemozoin assisted in trafficking parasite DNA to intracellular endosomes, and activated DCs via a TLR9- and MyD88-dependent signaling pathway (136). Hemozoin in isolation bound strongly to the murine TLR9 ectodomain while β-hematin, a synthetic form of hemozoin, was unable to activate DCs in this study (136). A subsequent study in humans did observe CD80, CD83, and CXCR4 upregulation on human moDCs after β-hematin stimulation (132), but the β-hematin-induced DCs were unable to induce allogeneic T cell proliferation (132).

Uric acid, another toxic product of *P. falciparum* metabolism, has also been found to upregulate expression of CD80, CD86, and CD11c and to downregulate HLA-DR on purified human blood DCs (138). Uric acid also reportedly stimulated mast cells to produce high levels of the DC growth factor Flt3-L in mice (102). Interestingly, DNAse treatment of uric acid abrogated its activatory effects on DCs (138), similar to what is seen with hemozoin. While uric acid is known to drive inflammation during *Plasmodium* infection through activation of the inflammasome [reviewed in (139)], the role of the inflammasome in anti-malaria DC responses and activation has not been investigated.

Together these studies highlight an important role for *P. falciparum* DNA as an activatory ligand, particularly in activating pDCs and driving production of IFN-α (107). Since only the pDC subset in humans expresses TLR9 [reviewed in (140)], ligation of TLR9 by *P. falciparum* DNA and subsequent cytokine production by pDCs may be one of the primary mechanisms by which human DCs are activated by *Plasmodium* (107, 136). Cytoplasmic pattern recognition receptors for *Plasmodium* DNA, which are expressed in all DC subsets, may also play a prominent role in anti-*Plasmodium* interferon responses (141, 142). Of particular interest are the role of STING-dependent responses in DCs and their role in anti-malaria responses. Considering the wide distribution of *Plasmodium* DNA throughout the host during infection (143), the human DC response to malarial DNA *ex vivo* and *in vitro* is one of the key gaps in knowledge that remains unaddressed.

Murine studies have identified a number of other immunostimulatory *Plasmodium* products, but their role in DC activation has not yet been investigated. Glycosylphosphatidylinositol (GPI) molecules, membrane anchors for *Plasmodium* surface proteins, are known ligands for TLR2 (144), expressed on human cDCs (118). Parasite RNA is known to induce type I IFN via TLR7/MyD88-dependent signaling (145), and TLR7 is highly expressed on human pDCs (118), the major producers of type I IFN. Finally, microvesicles are small organelles of 1 μm of less in size, derived by blebbing of the plasma membrane, which are generated in high volumes during *Plasmodium* infection (146, 147). They can contain a range of parasite material, and are able to induce cytokine secretion from murine (148) and human (149) macrophages. In summary, considering the wide range of immunostimulatory molecules produced by *Plasmodium* spp., it remains interesting that the DC response to malaria is not always activatory. While further studies should continue to identify *Plasmodium* ligands that drive DC activation, this must be studied in combination with the factors that underlie DC suppression in malaria.

DCS AND *PLASMODIUM*: OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

Immunity to *Plasmodium* is complex, and many aspects of cellular immunity remain poorly understood, particularly the impact of malaria on DC function. A better understanding of DC responses to *Plasmodium* will provide insight into the low efficacy and relative short duration of protection of the current malaria vaccine RTS,S, as well as the slow acquisition of natural immunity in malaria-exposed individuals. One of the most important unresolved questions is one of DC "suppression": namely, whether exposure to *Plasmodium* spp., particularly *P. falciparum*, inhibits the ability of DCs to initiate and orchestrate effective immune responses. Determining precisely how DCs are modulated by *P. falciparum* is crucial for understanding the development of immunity to malaria.

Some of the most interesting insights into the effects of malaria-induced DC impairment come from field studies that stratified patients by severity of infection. Studies that analyzed mild vs. severe malaria cases separately did not observe significant differences in DC phenotype between the groups (82, 102), suggesting that there may be a "tipping point" beyond which DC dysfunction is altered regardless of the severity of clinical presentation. This is underscored by the similarities in DC phenotype between natural exposure and CHMI. The latter are a naïve population, but during acute infection and for at least 24 h after treatment they exhibited similar DC phenotypes to those seen in naturally infected cohorts. Repeated infections could lead to sustained downregulation of DC function. Considering that successful induction of vaccine responses requires DC involvement, the failure of multiple malaria vaccines when transitioning from naïve populations to endemic populations (21, 150) may be due to impaired DC function in these endemic populations prior to vaccination.

Some valuable insights could be obtained by investigating differences in DC impairment between asymptomatic and symptomatic malaria cases. The systemic inflammation characteristic of symptomatic malaria undoubtedly contributes to DC dysfunction. While asymptomatic cases seem to experience similar loss of DC function short-term (87, 88, 99), it may be that they exhibit better recovery of DC function long-term. The phenotypic differences between high- and low-dose inoculation cohorts in CHMI studies (89, 91, 92) suggest that administering curative treatment while parasitaemia is still very low is also effective at limiting DC impairment. The similarities between naturally infected cohorts (**Figure 3**) also suggest that transmission intensity does not have a major impact on DC dysfunction beyond a certain threshold. Studies that follow the long-term effects of malaria exposure on DC function will be essential to clarify whether DC impairment persists after malaria elimination, especially in unstable transmission settings.

Variations in methodology have made it difficult to ascertain the effects *Plasmodium* parasites have on DCs *in vitro*. Particularly, care must be taken when stating that *P. falciparum* "suppresses" DC function *in vitro*: levels of activation lower than that seen in response to positive controls is not necessarily indicative of suppression. True suppression should be defined by an inability of DCs to become activated by known activatory stimuli, particularly pattern recognition receptor ligands. Analyses should also always account for increased cell death, which may result in false reports of suppression. Overall, the data indicates that DC function is not universally suppressed (**Figure 3**). Rather, *Plasmodium* appears to target specific pathways, among them the ones crucial for inducing

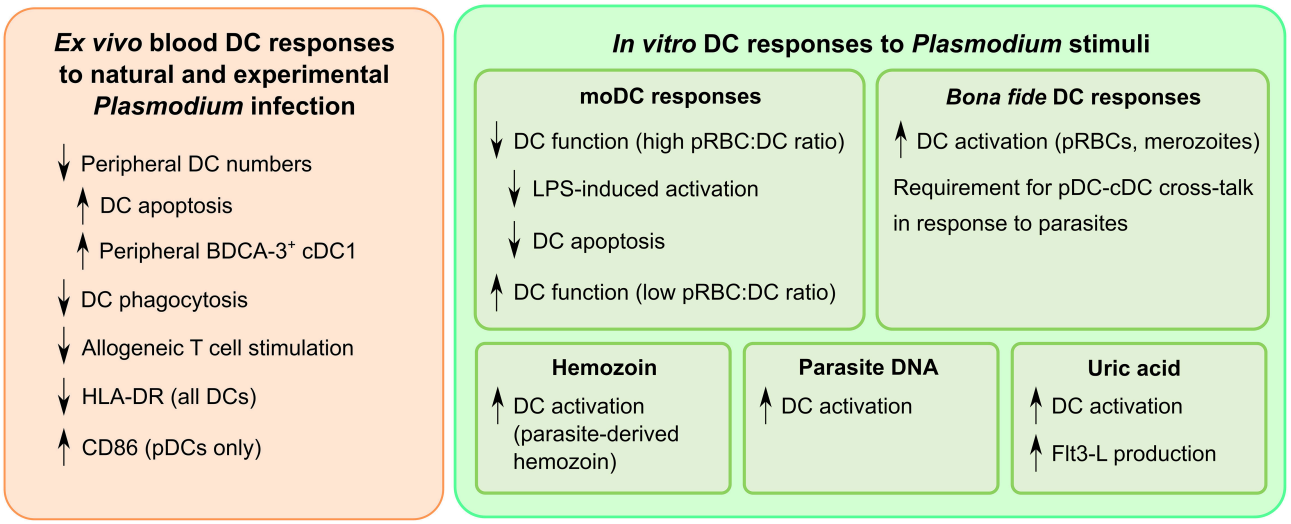

FIGURE 3 | A summary of dendritic cell responses to *Plasmodium*. While the dendritic cell response is heterogeneous, certain trends are evident when examining the entirety of the current literature. Downregulation of dendritic cell function is commonly observed in field studies of infected humans. *In vitro* studies have yielded insight into the complexities of dendritic cell activation by *Plasmodium*, particularly the types of ligands that can trigger an inflammatory response.

naïve T cell proliferation (82, 103, 104), while still allowing DCs to polarize responses toward a T_H1-like phenotype (85, 106). Research into whether the functionality of these T cells is affected relative to other pathogens which induce T_H1 polarization will be important to understand the uniquely *Plasmodium* factors that delay the development of antimalarial immunity.

There also appears to be an important role for DC cross-talk between pDC and cDC subsets (85, 107). This has two crucial implications: first, it warrants further study of how individual DC subsets respond to *Plasmodium* spp. without grouping them into a monolithic conglomeration of HLA-$DR^{hi}Lin^{-}$ cells. In particular, elevation of the circulating BDCA-3^{+} cDC1 subset is a commonly reported phenomenon in field studies but, due to the low frequencies of this rare population, its function in malaria has not been extensively investigated. More rigorous strategies, including cell sorting, should be employed to study how purified subsets respond to malaria. Secondly, pDC cross-talk is essential for production of cytokines such as CXCL10 and IFN-α. CXCL10, also known as IP-10, is majorly implicated in malaria pathogenesis (151–153), and IFN-α has recently been shown to downregulate antimalarial immunity (154, 155). As pDC help is required for production of both of these cytokines, and pDCs are the major producers of IFN-α in malaria (65, 155), strategies to reduce pDC activation in malaria might be beneficial for the longevity of antimalarial immunity. Moreover, malarial DNA has proven to be a potent inflammatory ligand (107, 136), and since only pDCs express TLR9 in humans (118), detection of malarial DNA by pDCs may play a significant role in detrimental cytokine responses, making pDCs an ideal target for strategies to reduce pathogenicity.

Moreover, *ex vivo* and *in vitro* studies depict a complex effect of malaria on the pDC subset. Numbers of circulating pDCs were reduced during natural infection (83, 84, 86, 97, 99) and CHMI (92, 95), though it is unclear whether this is due to pDC death or sequestration. One murine study indicated that pDC could be infected with or endocytose parasites (156). Whether a similar situation exists for human pDC is not known, but it is plausible that large numbers of parasites within pDC could kill these cells, leading to lower circulating numbers. Malaria infection also triggers the upregulation of CCR7 on pDCs (83), suggesting that homing to lymphoid tissues is enhanced during infection. Thus, circulating pDCs may not be the subsets responding to infection, which could explain why pDC activation has not been reported in most field studies. Multiple other factors could also contribute to this perceived lack of pDC activation. Firstly, sustained parasitaemia in malaria-endemic regions may downregulate expression of activatory ligands on circulating pDCs. Secondly, non-conservative gating of pDCs may misrepresent the activation state of this DC population. Thirdly, no field studies to date have measured IFN-α production, which is a direct and functional read-out of pDC activation. *In vitro* studies have observed IFN-α production when pDCs were directly stimulated with parasite products (83, 85, 107). This reinforces the need to study pDC function during malaria to understand whether a loss of pDC numbers in the periphery is associated with a concomitant loss of function.

When interpreting existing literature, it must be kept in mind that the majority of studies have focused on pRBCs and only a minority have examined responses to extracellular forms of the parasite such as merozoites. Considering that DC responses change depending on the parasite life stage (85, 105, 107), even within the relatively limited scope of the blood stages, it will be vital to study the differences between responses to each life stage. Since it is unlikely that a single vaccine will be able to target the entire *Plasmodium* life cycle, understanding the type of responses induced by each life stage is essential for designing new

TABLE 3 | Research priorities in DCs and malaria.

Priority	Approach
Understanding DC functionality to improve malaria vaccines	*In vitro* assays to understand which DC signaling pathways are activated or unaffected by malaria and exploit adjuvant technologies that target these pathways
	Incorporating DC functional assays into vaccine trials as a measure of vaccine relevance and functionality
Correlating DC function in malaria to protection	Controlled human malaria infection studies in naïve and previously exposed cohorts to understand how DC responses are altered by prior exposure and how this correlates with clinical immunity
	In-depth data analyses of how changes in DC phenotypes correlate with protective immune responses and/or overall clinical immunity
Understanding the mechanism of DC modulation by *Plasmodium* spp.	Development of small animal or *in vitro* models to assess human DC responses
	Thorough mapping of the functional and transcriptional changes that DC undergo upon encountering *Plasmodium* spp.
	Measuring DC responses to different *Plasmodium* life stages and determining which life stages have the greatest immunostimulatory potential to facilitate vaccine development

therapeutic interventions to protect the host against damaging immunopathology. A two-pronged "big data" approach could be particularly informative, with the use of RNAseq or proteomics on the DC side to examine immune pathways induced by each parasite life stage, and conversely a proteomics or other—omics-based approach examining the potential immunogens expressed by each parasite life stage. In particular, understanding the pattern recognition receptor signaling pathways which are inhibited or activated by pRBCs would enable more targeted therapies to reverse their suppressive effects. This would inform functional studies, and therefore form a roadmap for vaccine strategies or other therapeutic approaches that could induce potent, long-lasting antimalarial immunity.

A primary caveat of the current literature on human DC responses to malaria, particularly in field studies of infected individuals, is that all studies have looked at circulating blood DCs. It may be that mature DCs migrate into the tissues while immature DCs remain in circulation. Therefore, care should be taken not to generalize the phenotype of these circulating DCs to the responses of liver, spleen or bone marrow tissue-resident DCs, which may have greater functional relevance. Understandably, obtaining tissue-resident cells directly from humans is difficult and ethically challenging. Thus, models such as humanized mice, which produce DCs functionally similar to those found in humans (57, 157), are a promising system to study DCs with a tissue-resident phenotype. While a humanized mouse that is able to support the entire *Plasmodium* life cycle is still out of reach, recent technological improvements have enabled development of models that allow study of immunity to specific life stages [reviewed in (158)]. For example, humanized liver mice could shed light on the elusive phenomenon of liver-stage immunity, while mice with humanized immune systems could provide better insight into cell-mediated mechanisms of protection against the blood stage. Development of a complete humanized mouse model for *Plasmodium* would be invaluable for human immunological research and vaccine development.

Organoids, miniature models of organ function, have proven useful in studying tissues such as the liver (159) and intestine (160). An intestinal organoid model has already been used to study transcriptomic regulation of another Apicomplexan with a complex life cycle, *Cryptosporidium* (161). Development of a splenic organoid could be used for development of functional tissue DCs and enable further study of blood-stage malaria, as well as other blood-borne diseases (162). Liver and skin organoids would also be invaluable for studying the pre-symptomatic phase of the life cycle, and aid development of a vaccine that confers sterile immunity.

Finally, data from *in vitro* studies using moDCs as a model may not be representative of the interactions between *Plasmodium* and steady-state DCs. Both moDCs and *bona fide* DCs show pro- and non-inflammatory responses to *Plasmodium* stimulation, but phenotypic and transcriptomic differences between them highlight that the moDC phenotype is pronouncedly different and may not necessarily be generalizable (125). It was previously thought that moDCs might be analogous to $CD16^+$ DCs. However, recent findings outline that moDCs and $CD16^+$ DCs exist as separate populations in the steady-state (124, 125), and while they may have convergent functions during inflammation, this has not yet been conclusively shown. Therefore, caution should be taken when describing DC-parasite interactions using results generated with moDCs: while it is likely that moDC-like APCs are generated during the course of *Plasmodium* infection, moDCs may not accurately reflect the behavior of steady-state DC populations.

This review has outlined many facets of DC function in malaria that are not well understood (**Table 3**). Firstly, DCs from naïve individuals appear to respond differently to each malaria life stage. These responses should be compared to those seen in exposed individuals for a better understanding of how prolonged malaria exposure affects immune recognition. Secondly, we must further develop an understanding of how the DC phenotypes we observe *ex vivo* and *in vitro* translate into effectiveness, duration, and quality of antimalarial responses. Including DC studies in vaccine trials would help to address which elements best describe a beneficial DC response. This should be supplemented by more studies of DCs in natural infection against comparable non-exposed donors. Thirdly, there is a need for better models to examine DC function in malaria. DC studies of the future should focus as much as possible upon *bona fide* DCs, and seek to

develop new models that will permit a more in-depth study of how DC function is altered by the malaria parasite.

To conclude, the complexities of DCs make them a relatively understudied cell type in the context of malaria, where they have a potentially pivotal role in the regulation of antimalarial immunity. Many gaps in knowledge remain to be addressed, and there is a prominent need for novel technologies to bridge the gap. A deeper, more rigorous understanding of how *ex vivo* and *in vitro Plasmodium*-exposed DC phenotypes correlate with effective immunity, and the mechanisms that regulate DC interactions with *Plasmodium* will grant valuable insight into the acquisition of immunity, and form a basis for the development of better vaccines.

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XZY wrote the first draft of the manuscript, which was reviewed and edited by RJL, JGB, and MOK. XZY and RJL prepared tables and figures. All authors have read and revised the manuscript.

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Different Life Cycle Stages of *Plasmodium falciparum* Induce Contrasting Responses in Dendritic Cells

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Dendritic cells are key linkers of innate and adaptive immunity. Efficient dendritic cell activation is central to the acquisition of immunity and the efficacy of vaccines. Understanding how dendritic cells are affected by *Plasmodium falciparum* blood-stage parasites will help to understand how immunity is acquired and maintained, and how vaccine responses may be impacted by malaria infection or exposure. This study investigates the response of dendritic cells to two different life stages of the malaria parasite, parasitized red blood cells and merozoites, using a murine model. We demonstrate that the dendritic cell responses to merozoites are robust whereas dendritic cell activation, particularly CD40 and pro-inflammatory cytokine expression, is compromised in the presence of freshly isolated parasitized red blood cells. The mechanism of dendritic cell suppression by parasitized red blood cells is host red cell membrane-independent. Furthermore, we show that cryopreserved parasitized red blood cells have a substantially reduced capacity for dendritic cell activation.

Keywords: dendritic cell (DC), malaria, malaria vaccines, parasite-host interactions, innate immunity

INTRODUCTION

Dendritic cells (DCs) are key linkers of innate and adaptive immunity and their activation is essential to vaccination. Ablating DCs in murine models leads to vaccination failure and subsequent loss of antigen-specific T cells (1). Immunization of individuals living in malaria-infested regions has been notoriously difficult, with even the most promising vaccines failing to provide high levels of efficacy. A failure to activate DCs in people infected with *Plasmodium falciparum* blood-stage parasites may provide some explanation to the low efficacy of malaria vaccines [reviewed in (2)]. It may also explain the slow acquisition of natural anti-malarial immunity in individuals residing in malaria-endemic settings.

Activation of DCs is required for their subsequent efficient activation of T cells. Activated DCs increase surface expression of the co-stimulatory markers MHCII, CD40, CD80, and CD86, which are all involved in T cell co-stimulation. In particular, the interaction between CD40 on DC and its ligand CD40L on T cells is integral to enhance both T cell and DC responses for effective adaptive immune responses (3).

There remains an unresolved question of whether exposure to *P. falciparum* inhibits DC activation, leading to DC "suppression." Literature from *ex vivo* studies has suggested that DCs from infected individuals are inhibited in core DC functions, namely: expression of co-stimulatory markers, cytokine secretion, antigen uptake, and the ability to induce primary and secondary T cell responses (4–7). However, there is a lack of consensus among *in vitro* studies as to what effect *P. falciparum* has directly upon DCs. To date the majority of literature has examined DC responses to parasitized erythrocytes (pRBCs), the intracellular stage of the asexual parasite. In this life stage *P. falciparum* is concealed from host immunity by the host erythrocyte membrane, though parasite proteins such as the adhesion ligand *PfEMP1* are exported to the erythrocyte surface. Very little research into cellular responses to the infectious extracellular stage, known as the merozoite, has been carried out. Furthermore, it is important to note that the majority of *in vitro* studies have been carried out in monocyte-derived (mo) DCs (8), which are phenotypically and functionally different from *bona fide* DCs (8, 9).

The murine Flt3 ligand-induced DCs (FL-DCs) closely recapitulate the DCs of the blood, similar to spleen DC, but slightly less mature conventional (cDC) and plasmacytoid (pDC) DC subsets (10–13). Therefore, the FL-DC model was used here for its ease and its capacity to generate large volumes of *bona fide* DCs, enabling us to test a broad variety of parameters.

The FL-DC cDCs can be divided into two subsets by expression of CD24 and CD11b. The FL-DC $CD24^+$ cDCs ($CD24^+CD11b^-$) are immature cDC1. The cDC1 subset is specialized in cross-presentation and has a superior ability to prime naive cytotoxic $CD8^+$ T cells, and polarize T cells toward the T_H1 pathway via IL-12 production. Meanwhile, the FL-DC $CD11b^+$ subset ($CD24^-CD11b^+$) is equivalent to immature cDC2 (11). These FL-DC cDC1 and cDC2 populations share many pattern recognition receptors with their human blood DC counterparts and the core surface phenotypes and functions of cross-presentation (cDC1) and bacterial recognition and $CD4^+$ T cell stimulation (cDC2) are conserved [reviewed in Macri et al. (14)].

In contrast, pDCs are overall poor stimulators of T cell responses and do not upregulate co-stimulatory markers. Their defining characteristic is the secretion of Type I and III interferons (IFNs) in response to viral infection and/or the ligation of endosomal TLRs (15–17). In humans, pDCs are also the only subset to express TLR9, which is a known receptor for malarial DNA (18, 19).

Previous literature has described pDC production of IFN-α in response to *P. falciparum* merozoites *in vitro* (19) and during natural infection in humans (20), where pDCs appear suppressed in the presence of malaria parasites (21). Further studies hinted at pDC as a source of replicating malaria parasites (22), but this subset has, overall, been poorly studied in malaria.

This study aimed to identify how DC responses to the pRBC and the merozoite differ, with findings indicating that merozoites induce DC activation whereas pRBCs induce low co-stimulatory marker expression but high production of cytokines, with greater suppression at increasing doses of pRBCs. We reveal that the mechanism of suppression of DC activation occurs independently of the host RBC membrane, is not seen with pRBCs that have undergone a freeze-thaw cycle, and cannot be rescued by merozoites nor an exogenous TLR9 stimulus.

METHODS

Culture, Purification, and Freeze-Thawing of *P. falciparum* Blood-Stage Parasites

Plasmodium falciparum 3D7 parasites were cultured as per standard protocols. Briefly, parasites were maintained in RPMI1640 (Gibco) enriched with 5% Albumax (Gibco) and 5% AB^+ human serum obtained from the Red Cross, Melbourne, Australia. Parasites were cultured at 3–5% haematocrit using type-O negative blood (Red Cross) and incubated in medical gas (1% O_2 and 5% CO_2 in nitrogen gas) at 37°C as per standard protocols. Parasite growth was assessed daily by thin smear (23) stained with Giemsa's azur eosin methylene blue solution (Merck Millipore). Parasites were sorbitol synchronized (24) and selected for knob expression by gelatin flotation (25). All parasites were initially treated with *Mycoplasma* removal agent (MP Biomedicals) for 2 weeks as per the manufacturer's instructions. Subsequently, parasites were tested at 1-monthly intervals for *Mycoplasma* contamination using the MycoAlert test kit (Lonza).

Trophozoite Preparation

Trophozoites at 24–28 h post-invasion were isolated by magnetic selection with the VarioMACS (Miltenyi Biotech) system. Purity was assessed by counting 200 cells via Giemsa thin smear. Preparations varied from 70 to 99% parasite purity and preparations with purity of <90% were re-purified by repeating magnet selection. Purified preparations were counted using a haemocytometer and resuspended to the appropriate cell concentration in complete medium (CM): RPMI1640 containing GlutaMAX (Gibco) supplemented with 10% heat-inactivated FCS, 0.1 mM 2-beta-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin. Aliquots of purified trophozoites with >95% purity were also frozen in glycerolyte 57 (Baxter Healthcare Corporation) and stored at −80°C to compare efficacy of DC stimulation with same-day isolated cultures.

Merozoite Preparation

Merozoites were purified as per Boyle et al. (26). Briefly, late-stage (30–34 h) trophozoites were purified and returned to culture without uninfected RBCs (uRBCs). When trophozoites had matured into schizonts (40 h), 10 μM of protease inhibitor E64 (Sigma) was added to culture medium to inhibit merozoite egress. Upon maturation into late-stage schizonts (46–48 h), pRBCs were resuspended in 20 mL of RPMI-HEPES and schizont rupture was induced by pushing through a 1.2 μM syringe filter (Pall), resulting in purified merozoites. Merozoites were stained with ethidium bromide (EtBr) and counted by flow cytometry. For storage, late-stage segmented schizonts derived by this method were frozen in PBS at a concentration of 150×10^7/mL after arrest with E64 but prior to filter lysis and stored at −80°C until use in assays.

Saponin Lysis to Remove Host RBC Membranes

Magnet-purified pRBCs or empty "mock" tubes were co-incubated with 0.09% saponin in 1X PBS for 30 s at RT, after which they were washed three times in RPMI containing 2% FCS to remove residual saponin. Saponin lysis of host RBC membrane was confirmed by microscopy. Both saponin-treated and intact parasites were then resuspended in CM and co-incubated with FL-DCs in the presence or absence of 0.5 μM CpG2216. CM or CM and CpG2216 were added to "mock" tubes to control for any potential carry-over of the detergent and added to FL-DCs as separate controls.

FL-DC Culture

Bone marrow cells were stimulated with Flt3-L to induce large quantities of DCs similar in phenotype and function to those found in murine spleen (11, 12, 27) (**Supplementary Figure 1**). Briefly, FL-DCs were obtained as follows: leg bones of C57Bl/6 mice (AMREP Animal Services Precinct Animal Centre, Melbourne, Australia) were dissected out and harvested into RPMI containing 2% v/v FCS. Bones were cut open and bone marrow was flushed with a 22 g needle using 2 mL of RPMI-FCS per bone. After centrifugation at 600 × *g* for 7 min, cells were co-incubated with 1 mL of red cell lysis buffer (Sigma) with constant mixing for 30 s. Cells were washed twice in RPMI1640 containing 2% FCS before resuspending in complete medium and filtering through a 70 μM sieve (Falcon). Cells were resuspended at 1.5×10^6 cells/mL in complete medium containing 100 ng/mL of Flt3-L-Ig (BioXCell) and cultured in vented cap tissue culture flasks (Corning) in a 37°C incubator in 5% CO_2 for 8 days.

Determining the Effect of the Parasite-DC Ratio Upon the DC Response to Purified Blood-Stage *P. falciparum*

After 8 days culture FL-DC were counted and washed in CM. Merozoites and pRBCs were titrated against FL-DCs in round-bottom 96-well-tissue culture plates (Falcon) at a final cell concentration of 0.5×10^6 FL-DC/mL. Additionally, FL-DCs were also cultured with 0.5 μM CpG2216 alone or in addition to parasites, or with media alone or uRBCs as negative controls. All incubations were carried out for 18–20 h at 37°C in 5% CO_2.

Flow Cytometric Analysis

For analysis of surface phenotype, cells were stained in 1X PBS supplemented with 2% v/v FCS and 10% v/v 0.1M EDTA (MACS buffer) with monoclonal antibodies for MHCII, CD11b, CD11c, CD24, CD45R, and CD199. For staining, antibody was diluted in MACS buffer and antibody cocktail was added at $10\mu L/10^6$ cells, with a minimum of 30 μL of cocktail per well or tube, and incubated in the dark at 4°C for 20 min. Cells were washed to remove unbound antibody and analyzed on a BD Fortessa or BD LSRII flow cytometer. DCs were identified as medium-sized cells on forward and side scatter and doublets and PI-positive dead cells excluded. Activation of viable DCs was measured by expression of costimulatory markers MHCII, CD40, CD69, CD80, and CD86 on each DC subset using antibodies outlined in **Supplementary Table 1**.

Cytokine Analysis

Frozen supernatants derived from parasite-FL-DC co-culture experiments were tested for the presence of IFN-γ, IL-1β, IL-2; IL-4, IL-5, IL-9, IL-12p70, IL-13, IL-17a, IL-18, IL-22, IL-23, IL-27, GM-CSF, IP-10, eotaxin, and MIP-1α by ProcartaPlex (eBioscience) multiplex as per manufacturer's instructions. Presence of MIP-2, IL-6, IL-10, GRO-α, MCP-1, MCP-3, MIP-1β, RANTES, and TNF-α were assayed for by both FlowCytomix (eBioScience) and ProcartaPlex. Expression of IFN-α and IFN-β was measured by a separate multiplex (ProcartaPlex).

RESULTS

The Ratio of pRBCs-to-DCs Affects Expression of DC Maturation Markers and This Is Not Rescued in the Presence of TLR9 Ligand

People in endemic malaria regions can have a wide range of parasitemia in peripheral blood. Those with asymptomatic malaria typically have lower levels of parasitemia, ∼5,000 *P. falciparum* parasites per mL of peripheral blood on average (28), depending on the setting. This equates to a ratio of ∼0.25 parasites to 1 DC in blood. However, significant numbers of asymptomatic people and those with symptomatic malaria have infections over 10^6 parasites per mL, equating to ∼50:1 ratio of parasites to blood DC (29, 30).

To model the effect of different parasite concentrations on blood DC we analyzed the response of murine FL-DC to purified pRBCs from a high ratio of 50:1 to a low ratio of 1:1. In replicate, CpG2216 was added to the cultures to ask whether the presence of pRBCs affected the activation of FL-DC by an additional stimuli. The addition of uRBC alone were used to control for responses to RBC alone. To determine cell activation, MHCII and CD40 and CD86 co-stimulation molecule expression was measured upon FL-DCs (**Figure 1**). Analyses of total FL-DC indicated a reduction in CD40 expression at ratios of pRBC:DC of >12.5, and at lower concentrations in the additional presence of TLR9 ligand (CpG2216). Separate analyses of cDC and pDC subsets indicated that high pRBC concentrations led to a significant reduction of co-stimulation marker expression on both $CD24^+$ and $CD11b^+$ cDCs (**Figure 1C**) that was particularly strong for CD40, and also occurred in the additional presence of CpG2216 (**Figure 1A**). While DC viability decreased in response to 50:1 concentrations of pRBC alone, viability was not significantly decreased by pRBC in the presence of CpG yet co-stimulation molecule expression still decreased (**Figure 1B**). In response to low ratios of pRBCs of <12.5 pRBC:DC, cDC expression of co-stimulatory markers was comparable to that induced by CpG2216.

A more complex relationship between MHCII and co-stimulatory marker expression and pRBC ratio was observed in pDCs. Upon stimulation with low ratios of pRBC, pDCs

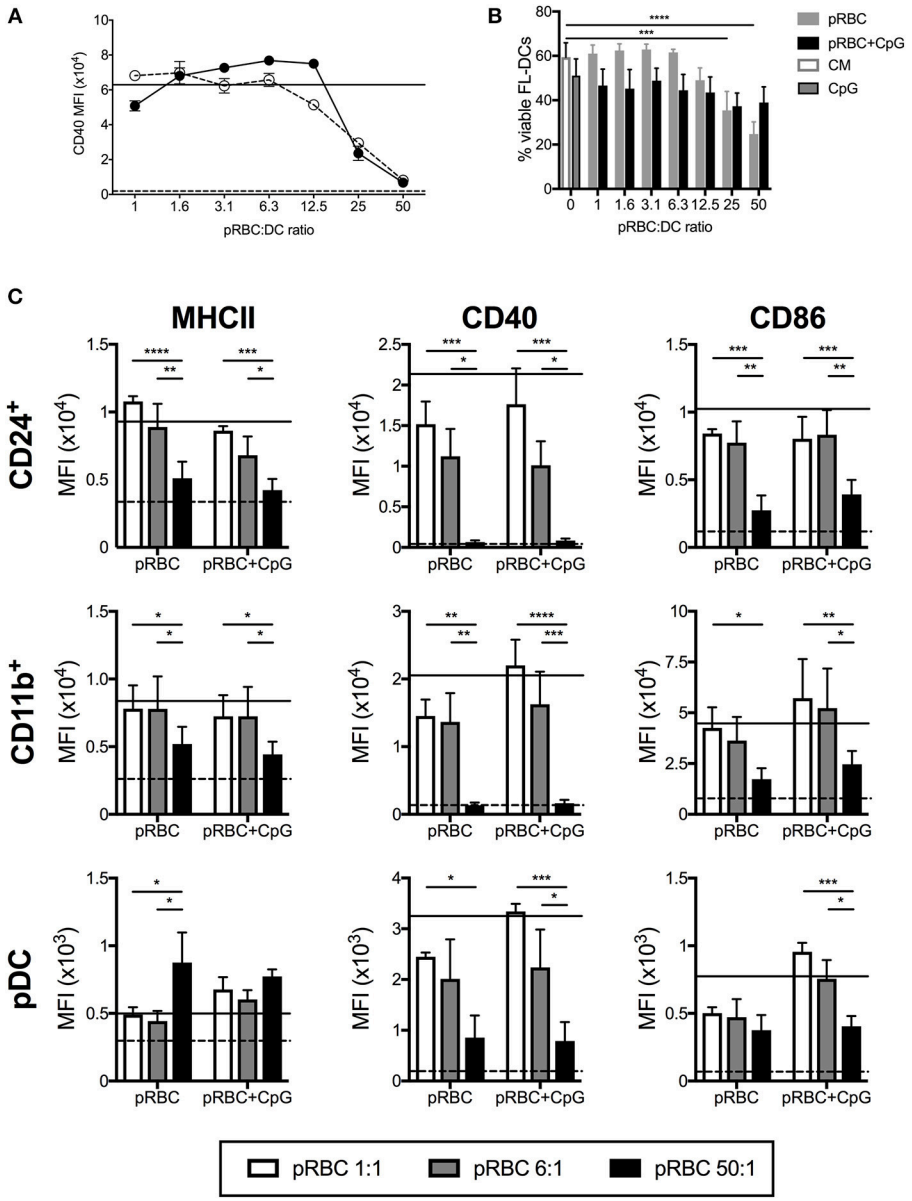

FIGURE 1 | High doses of pRBCs induce poor FL-DC activation and prevent normal TLR9-mediated activation. **(A)** Expression of CD40 on $CD24^+$ cDCs was downregulated in response to high doses of pRBCs alone (solid circles) or pRBCs + CpG2216 (hollow circles). Responses to CpG2216 alone (solid line) and uRBC alone (dashed line) are shown for comparison. **(B)** Viability of total FL-DCs in response to pRBCs alone (gray) or pRBCs + CpG2216 (black). Viability in response to culture medium alone (CM; white with gray border) or CpG2216 alone (CpG; gray with black border) are shown for comparison. **(C)** Expression of MHCII, CD40, and CD86 in response to pRBCs in $CD24^+$ cDC, $CD11b^+$ cDC, and pDCs. Co-stimulatory marker expression is shown in response to pRBC-to-DC ratios of 50:1 (black), 6:1 (gray), and 1:1 (white), as well as in response to CpG2216 (solid line) or uRBCs (dashed line). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. *P*-values were determined by two-way ANOVA using multiple comparisons. Data shown is from three experiments, each experiment using pooled FL-DC cultures from two mice. Error bars represent SEM of three separate experiments. MFI—geometric mean fluorescence intensity.

expressed MHCII, CD40, and CD86 at elevated levels compared to uRBCs alone, which could be further elevated in the presence of CpG2216. However, upon stimulation of pDC with pRBC ratios >25:1, in the presence or absence of CpG2216, CD40, and CD86 expression was reduced whereas MHCII (**Figure 1C**) and CD80 (data not shown) expression increased to levels above that seen with CpG2216 alone.

The Ratio of pRBCs-to-DCs Alters FL-DC Patterns of Cytokine Production Including in the Presence of TLR9 Ligand

The data of **Figure 1** indicated that at high pRBC:DC ratios, the response of DCs to CpG2216 was inhibited and that overall, DC activation was suppressed. To further investigate the activation of DC across a range of pRBC concentrations we analyzed the

production of cytokines in the supernatants of the cultured FL-DC.

Others have previously reported pDC-produced IFN-α in response to *P. falciparum* (19, 31, 32), with conflicting reports on whether the activation of pDCs was inhibited by pRBCs (19, 32). In the FL-DC cultures with pRBCs alone, IFN-α was only detectable in the cultures at ratios of <12.5 parasites per DC (**Figure 2A**). At pRBC:DC ratios of 1:1 and 6:1, we detected high levels of IL-6 and IP-10 (CXCL10) and detectable levels of TNF-α, IL-10, IFN-γ, and MIP-2 (CXCL2, **Figure 2B**). At pRBC-to-DC ratios of 50:1, IL-6, TNF-α and IL-10 production were reduced to the baseline levels induced by uRBCs whilst IP-10, IFN-γ and MIP-2 were produced at levels similar to those observed with ratios of 1:1 pRBC:DC (**Figure 2B**).

We then investigated whether cytokine secretion to pRBCs was boosted in the presence of TLR9 ligand, CpG2216, or if pRBCs were able to suppress CpG2216-induced cytokine secretion. The pDC and pDC-like cells are the only cells capable of producing high levels of IFN-α to CpG oligonucleotides, potent TLR-9 ligands (33). Addition of CpG2216 to pRBC-DC co-incubations increased overall cytokine secretion but a pRBC dose-dependent suppression of TLR9-induced IFN-α, i.e., pDC responses, was observed (**Figure 2A**) from bulk cultures. Reduced production of both TNF-α and IL-10 were also observed with pRBC:DC ratios of 6:1 and above (**Figure 2**). This suppression was marked at pRBC:DC ratios of 50:1 for IL-10, TNF-α, IL-6, and MIP2 production (**Figure 2B**).

Only Freshly Isolated pRBCs Are Suppressors of FL-DC Activation

We compared responses of FL-DCs stimulated with freshly isolated pRBCs and pRBCs previously purified and freeze-thawed. The protocol used for cryopreservation and thawing of pRBCs largely preserves pRBC integrity (34, 35) and is a convenient way of preparing standardized preparations of parasites for research, including experimental human infections (36, 37). However, frozen pRBCs were less efficient at activating the FL-DC (**Figure 3A** and data not shown). Moreover, they were inefficient at suppressing the TLR-9 activation of FL-DC compared to the freshly isolated pRBC (**Figure 3A** and **Supplementary Figure 2**). Therefore, frozen and fresh pRBC preparations were qualitatively different in their fundamental abilities to activate DCs.

Removal of Host RBC Membrane Does Not Prevent Parasite-Induced Suppression of DC Responses

The differences in the ability of fresh vs. frozen and thawed pRBC preparations to affect FL-DC activation suggested that perhaps a reduction in pRBC membrane viability was the cause. To test this, we treated freshly isolated pRBCs with the detergent saponin. Saponin treatment of pRBCs disrupts the RBC membrane and the parasitophorous vacuolar membrane (38). Thus, saponin-treated trophozoites lack expression of malarial proteins normally located on the host red cell membrane, including molecules such as *Pf* EMP1 which have been postulated to inhibit innate immune responses (39–41).

Co-stimulatory marker expression by DCs was similar after stimulation with saponin-treated and untreated pRBCs, with pRBC:DC ratios of 1:1 and 5:1 actually inducing lower levels of activation whilst high doses still led to low CD40 upregulation. In the presence of CpG2216, DCs were similarly activated, regardless of saponin treatment, whilst high doses of saponin-treated pRBC still suppressed the CpG2216-driven CD40 (**Figure 3**) and CD86 upregulation (**Supplementary Figures 3B,C**) on cDC and pDC populations.

Thus, neither the presence of host RBC membrane nor surface-expressed parasite proteins, including *PfEMP1*, appeared to play a major role in suppression of FL-DC activation in the presence of high levels of parasites. Viability was comparable between FL-DCs exposed to saponin-treated and non-treated pRBCs for every ratio of pRBCs to DCs (**Supplementary Figure 3A**). This data also suggested that the differences in DC activation abilities between frozen and freshly isolated pRBC were intrinsic to the trophozoite and not dependent on the host RBC membranes.

Merozoites Are Potent Stimulators of FL-DCs

Merozoites are the extracellular life stage of the malaria parasite. FL-DCs stimulated with ratios of merozoites to DCs from a high ratio of 50:1 to a low ratio of 1:1 were, in the main, highly activated, with dose-dependent increases of all surface activation markers examined (**Figure 4**), although $CD11b^{+}$ FL-DC showed reduced MHCII expression with high concentrations of merozoites (**Figure 4C**). However, in contrast to stimulation with pRBCs, at ratios higher than 12.5 merozoites:DC significant DC death was observed (**Figure 4B**). The rate of cell death at these ratios was high, consistent with excessive activation, and while cell surface activation of the remaining DC by TLR9 ligand was largely not perturbed by high concentrations of merozoites (**Figure 4C**), these results should be viewed in light of the very high rates of DC death at these parasite ratios.

FL-DC Production of Cytokines in Response to Merozoites Generally Increases With Higher Merozoite Dose

In response to increasing doses of merozoites, FL-DCs produced increasing amounts of cytokines (**Figures 5A,B**). This was inverse to the effect observed with pRBCs (**Figure 2**). Merozoites were a potent stimulus for IFN-α production. In the presence of CpG2216, production of TNF-α and IL-6 was reduced with increasing merozoites, potentially due to high merozoites concentrations inducing high DC death (**Figure 4B**). Overall, merozoites appeared to be potent activators of FL-DCs, inducing high levels of cell death at the highest concentrations but apparently not suppressing FL-DC activation by TLR9 ligands.

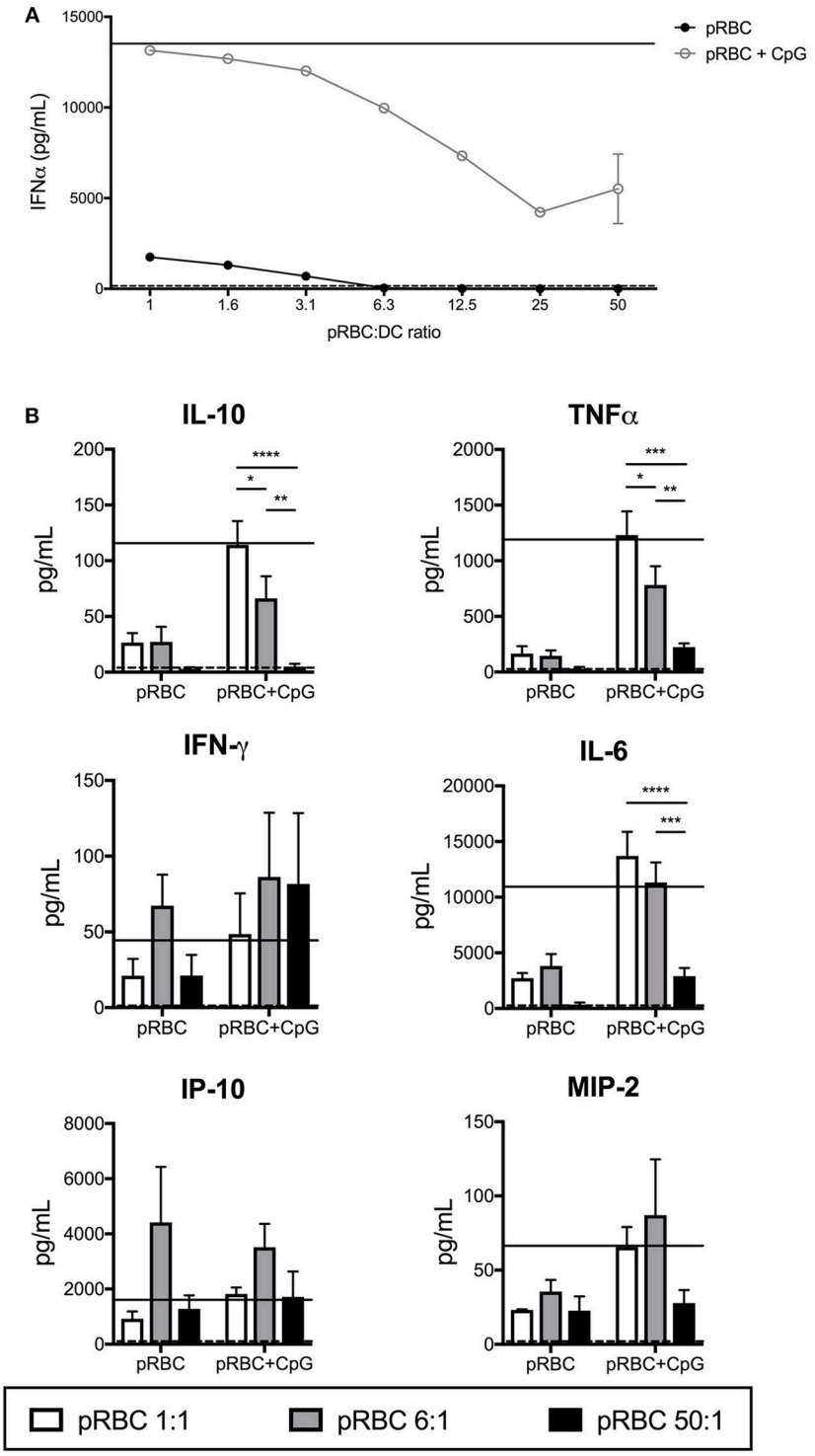

FIGURE 2 | Production of cytokines by FL-DCs is inhibited by high pRBC-to-DC ratios. Supernatants from FL-DC cultures stimulated with pRBCs were assayed for cytokine production using FlowCytomix and ProcartaPlex multiplexes. **(A)** IFN-α expression by FL-DCs was suppressed by the presence of high ratios of pRBCs, even in the presence of TLR9 ligand (pRBC+CpG). **(B)** FL-DC production of cytokines in response to pRBC-to-DC ratios of 1:1 (white), 6:1 (gray), and 50:1 (black) alone, or in the presence of CpG2216. Cytokine expression in response to uRBCs (dashed line) or CpG2216 alone (solid line) are included for comparison. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. *P*-values were determined by two-way ANOVA using multiple comparisons. Data shown is from three experiments with each experiment using pooled FL-DC cultures from two mice. Error bars represent SEM of three separate experiments.

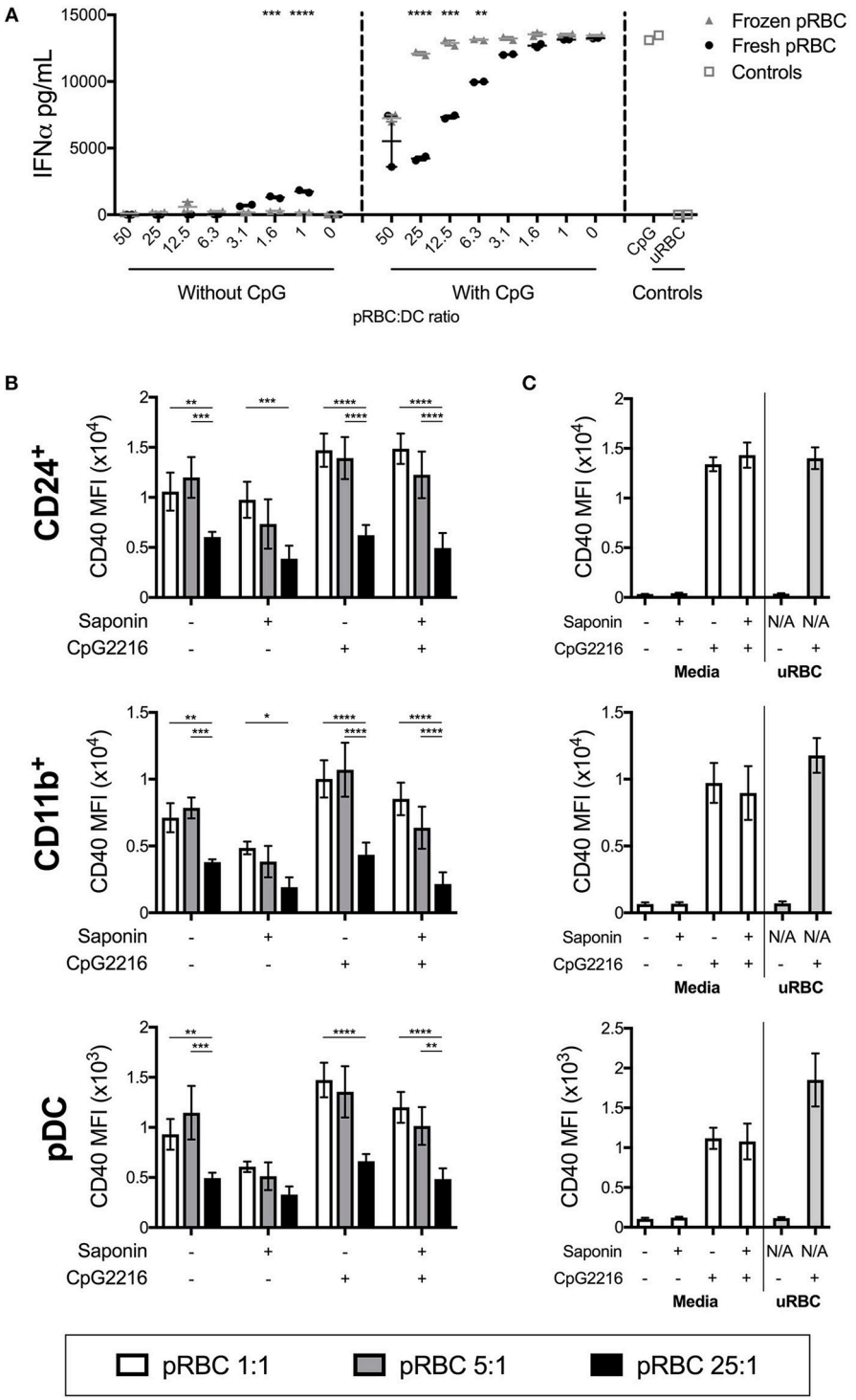

FIGURE 3 | pRBC-induced suppression is dependent on fresh parasites but not on intact host RBC membranes. **(A)** A titration of frozen pRBCs were compared to freshly purified pRBCs for the ability to induce activation and parasite-induced suppression of FL-DC TLR9 responses. Shown are IFN-α responses either to parasites alone or in the additional presence of CpG2216. **(B)** FL-DCs were co-incubated with saponin-treated or untreated *P. falciparum* pRBCs for 18–20 h at three different parasite-to-DC ratios: 1:1 (white), 5:1 (gray), and 25:1 (black), in the presence or absence of CpG2216. DC subpopulations were gated and expression of CD40 is shown. Each bar shows the SEM of three separate experiments, with each experiment using FL-DC cultures derived from pooling two mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. *P*-values were determined by two-way ANOVA using multiple comparisons. **(C)** To control for effects of any carry-over saponin, saponin was also added to empty tubes and washed alongside the pRBC samples. Culture medium alone or medium containing CpG2216 were aliquoted into these tubes (designated Saponin +) and used to stimulate FL-DCs. The lack of any suppression of CD40 expression by uRBCs alone (25:1) is shown.

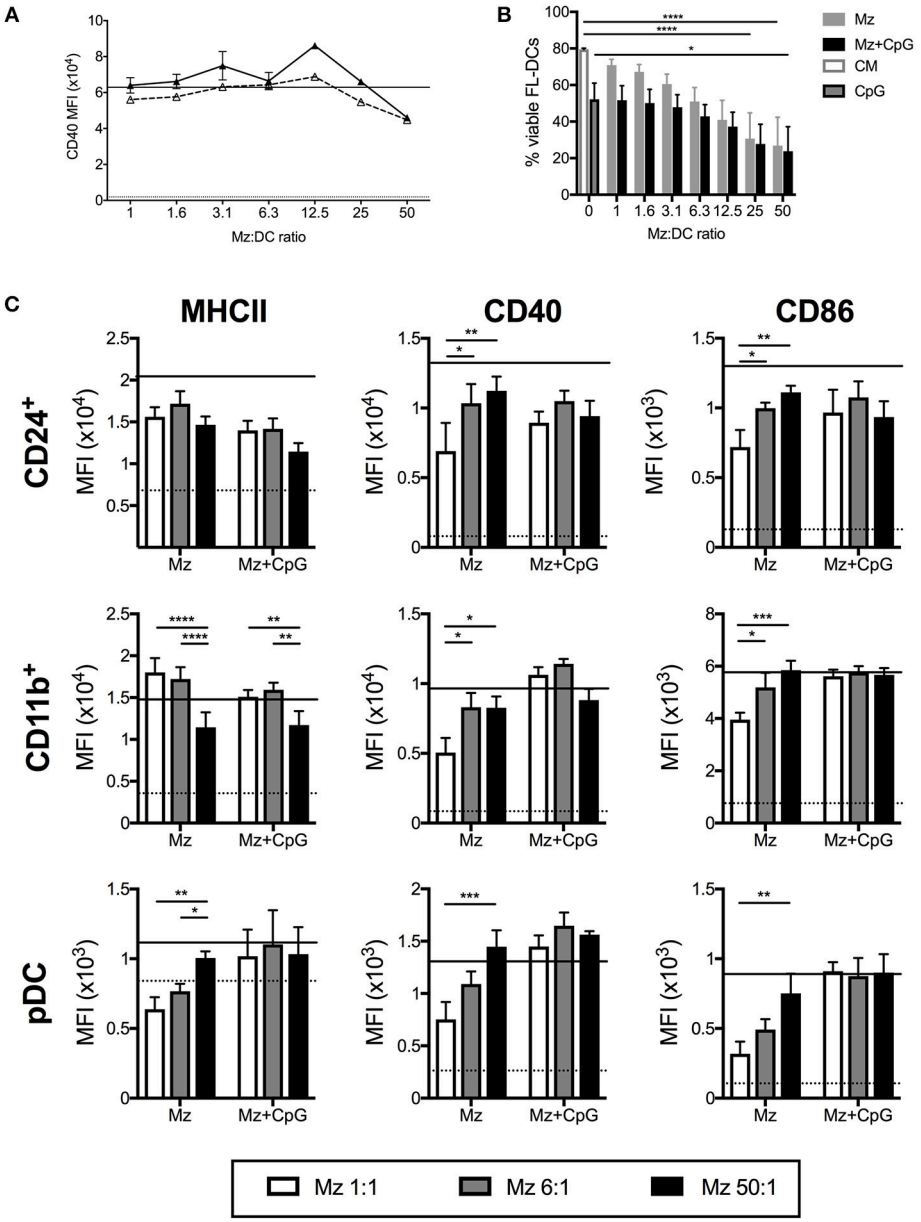

FIGURE 4 | Merozoites induce potent activation but high cell death of FL-DC. **(A)** Surface expression of CD40 on $CD24^+$ cDCs in response to 1–50 merozoites per DC (solid triangles) or in the additional presence of CpG2216 (hollow triangles). Level of CD40 in response to CpG2216 alone (solid line) and media alone (dotted line) are shown for comparison. **(B)** Exposure to high doses of merozoites results in death of up to 90% of FL-DCs after 18 h culture. Viability in response to culture medium alone (CM; white with gray border) or CpG2216 alone (CpG; gray with black border) are shown for comparison. **(C)** MHCII, CD40 and CD86 surface expression on FL-DCs gated for $CD24^+$, $CD11b^+$ or pDC in response to 1:1 (white bars), 6:1 (gray bars), or 50:1 (black bars) merozoites:DC, alone or in the addition presence of CpG2216.). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. *P*-values were determined by two-way ANOVA using multiple comparisons. Data shown is from three experiments, each experiment using pooled FL-DC cultures from two mice. Error bars represent SEM of three separate experiments.

Merozoites Cannot Rescue FL-DC Suppression Induced by pRBCs

During malaria infection DCs would encounter both trophozoite and merozoite life-stages of the malaria parasite. Since merozoites did not suppress DC activation we tested whether merozoites would rescue the upregulation of CD40 in the presence of pRBCs, with or without TLR9 ligand. A ratio of 25 pRBCs:DCs was used with the addition of equal numbers of merozoites. Ratios of 25 parasites per DC for each life stage were chosen as concentrations that were still inhibitory for pRBCs (**Figure 1A**) but maintained high CD40 levels in response to merozoites alone (**Figure 4A**). Whilst merozoites are potent stimulators of MHC and co-stimulatory molecule expression on FL-DCs (**Figure 4**), they were unable to efficiently activate

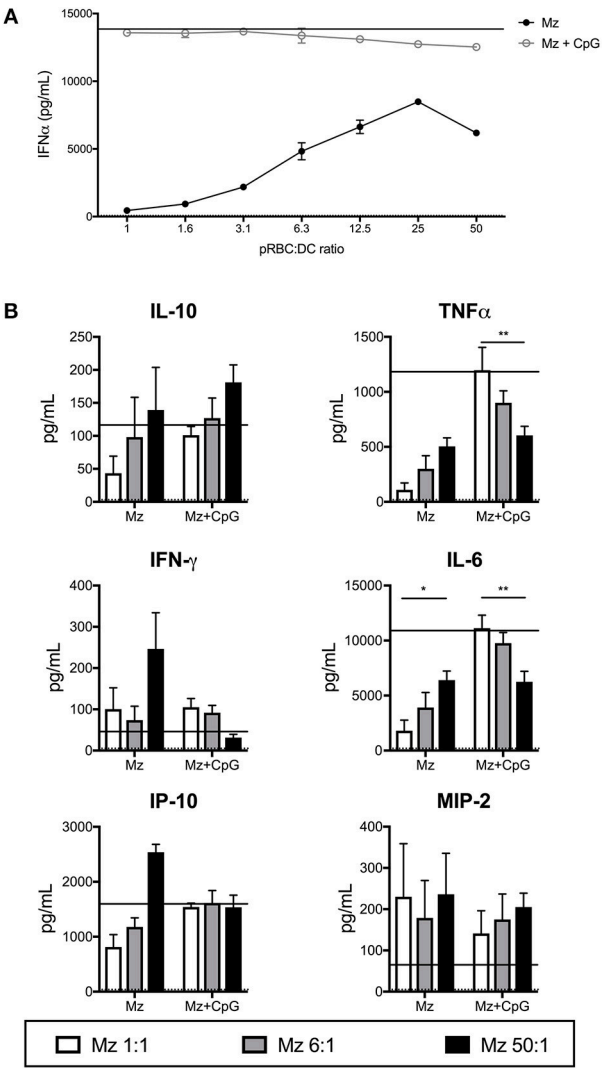

FIGURE 5 | Merozoites induce expression of a range of cytokines from FL-DCs. FL-DCs were incubated for 18 h with merozoites (Mz) and cytokines were measured in the supernatant using FlowCytomix and ProcartaPlex multiplexes. **(A)** High ratios of Mz:DCs induce high expression of IFN-α. **(B)** Mz:DC ratios of 1:1 (white bars), 6:1 (gray bars), or 50:1 (black bars). The expression in response to media alone (dotted line) and CpG2216 alone (solid line) were included for comparison. $^{*}p < 0.05$, $^{**}p < 0.01$. *P*-values were determined by two-way ANOVA using multiple comparisons. Figure shows pooled data from three experiments of FL-DC cultures derived from two mice each.

FL-DCs in the presence of pRBCs, particularly the upregulation of CD40 (**Figure 6**), either when mixed with pRBCs alone or in the additional presence of CpG2216 (**Figure 6**).

DISCUSSION

We have shown that at doses of >6 parasites to 1 DC, *P. falciparum* pigmented trophozoites possess a stage-specific capacity to suppress specific DC functions. This suppressive capacity particularly affects the upregulation of CD40 on all DC subsets and the ability of DCs to produce key cytokines; IFN-α, TNF-α, IL-6, and IL-10. This suppressive capacity of pRBCs is not dependent upon *Pf*EMP1 or other parasite molecules expressed on, or other properties of, the infected host RBC membrane (**Figure 3**).

The pRBCs not only suppressed DC responses to themselves at ratios of >6 per DC but also suppressed the ability of DCs to respond to the potent TLR9 ligand, CpG2216 (**Figure 1**). This included inhibiting the ability of pDCs to produce IFN-α to CpG2216 (**Figure 2A**). Although suppressing TLR9-mediated DC activation, increasing doses of pRBCs did not enhance DC death. Others have previously shown that monocyte-derived DCs, DCs fundamentally different to those investigated herein, also do not require *Pf*EMP1 to be suppressed, though suppression of DC activation at doses of 100:1 were at least in part due to DC apoptosis (42). The data shown herein of suppression of TLR9-mediated FL-DC activation by pRBCs is independent of cell death (**Figure 1**). In the aforementioned study, it was also shown via transwell experiments that cell-cell contact was not required for 100:1 doses of pRBCs to inhibit TLR-mediated upregulation of HLA-DR, CD83, and CD86 (42). Similarly, lysates from pRBCs induced chemokine production from human DCs, similarly to intact pRBCs (32). Both suppression and activation of DCs by pRBCs are therefore mediated by soluble factors, which could potentially be addressed by co-incubating TLR-stimulated DCs with conditioned parasite medium and measuring suppression.

In stark contrast, purified merozoites induced dose-responsive activation of the FL-DCs and high (likely activation-induced) DC death at high doses (**Figures 4**, **5**). The merozoites were not able to rescue the activation of FL-DCs in the presence of pRBCs; in fact, pRBCs inhibited the strong merozoite-driven DC activation (**Figure 6**). DC death in malaria is via the apoptotic pathway (4, 42, 43), and it is unclear whether the DC death observed in response to high ratios of parasites in this study is due to parasites directly activating pro-apoptotic signals or whether it is apoptosis as a natural endpoint of DC activation. The increased DC apoptosis may inhibit activation of surrounding DCs by impairing the maturation of immature DCs (44) and polarizing DCs toward a tolerising phenotype (45). Regardless, DC apoptosis is likely a major contributor to the short duration of immune memory to malaria. Determining whether apoptosis is driven by maturation or parasites will help to identify methods of reducing apoptosis and improve downstream DC responses.

In contrast, ratios of 1:1 pRBC potently activated DCs to upregulate co-stimulatory markers (**Figure 1**) and cytokines, including IFN-α (**Figure 3**). Merozoites on the other hand, really showed a dose response effect in their ability to activate DC (**Figures 4**, **5**), although ratios of 1 merozoite per DC still activated DC, highest levels of cytokines and cell surface markers were reached with more than 6 merozoites per DC. As merozoites and trophozoites have divergent transcriptomes (46, 47), this may help to identify potential activatory proteins. Furthermore, DC activation by these very low ratios of parasites suggests that even very low parasitaemia may induce potent DC activation, at least initially. Asymptomatic parasitaemia, often

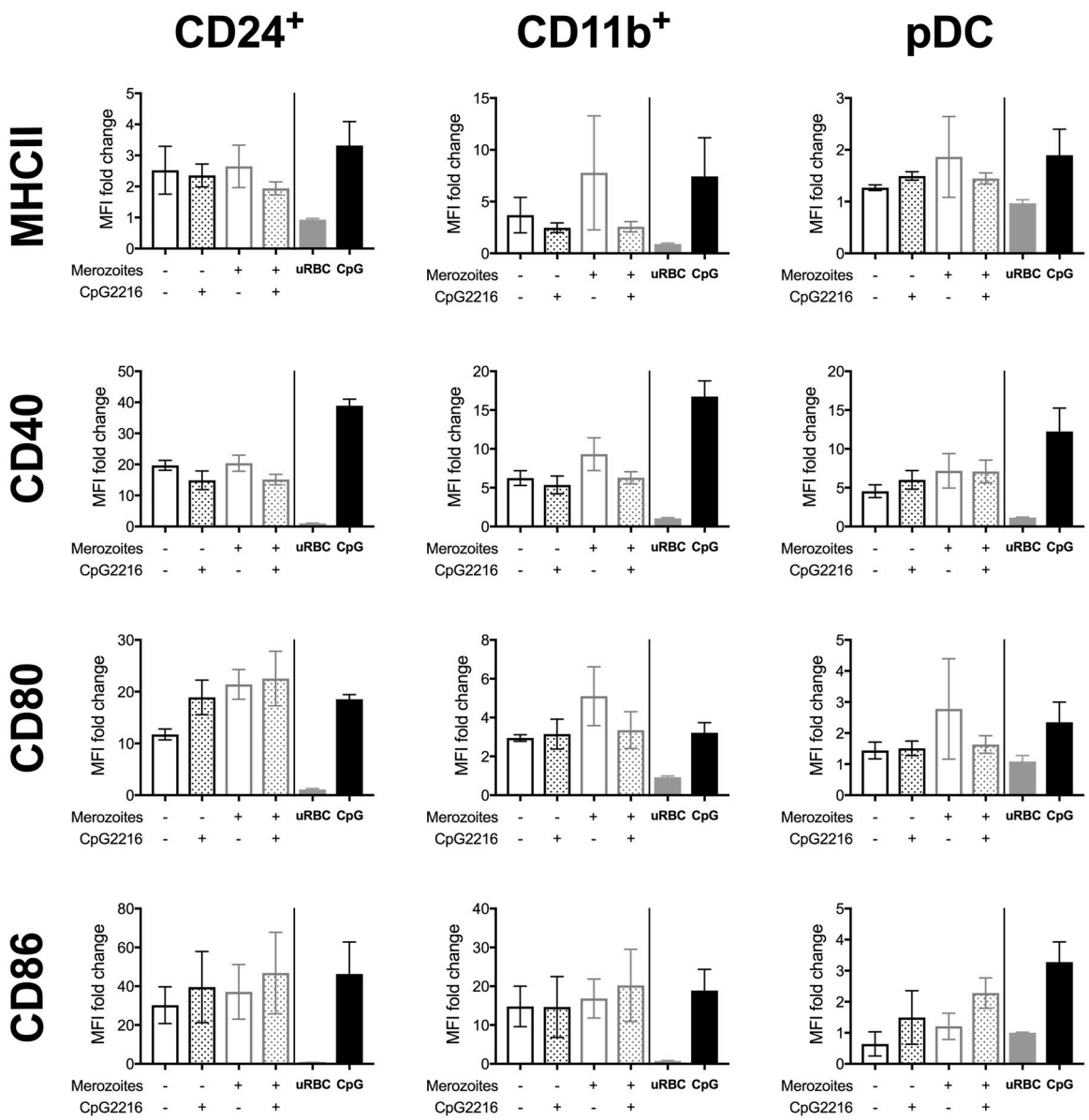

FIGURE 6 | Stimulation with mixed high-dose pRBCs and merozoites in the presence or absence of CpG2216 does not result in recovery of CD40 expression. FL-DCs were stimulated with either pRBCs alone, or pRBCs in the presence of an equivalent ratio of merozoites (Mz), in the presence or absence of CpG2216. All pRBCs and merozoites were added at a ratio of 25 per DC. Neither the addition of merozoites nor the addition of CpG2216 resulted in rescue of CD40 expression, but other markers demonstrated a heterogenous response. Co-stimulatory marker expression is represented as fold increase over media control and each bar represents mean and SEM of two experiments based on pooled FL-DC cultures of two mice.

seen in areas of high transmission, may reflect a pattern where initial potent DC-driven responses are suppressed by subsequent high-parasitaemia infections, leading to immunosuppression and tolerance. In contrast, low-parasitaemia infections may actually induce potent DC responses that lead to the generation of memory.

As regards physiological relevance of the parasite-to-DC ratios examined in this study, there is relatively little information on bioavailability of blood-stage malaria parasites to splenocytes *in vivo*. However, *in vitro* studies suggest that in high-parasitaemia settings a large proportion of merozoites fail to invade (26). This would result in high volumes of merozoites circulating to or being trafficked to the primary lymphoid tissues by migratory DCs, potentially even to the 50:1 ratios reported in this study. It is also observed *in vivo* that the spleen can remove parasites from RBCs without inducing RBC lysis (48–50), and while there is no information at what rate this occurs, splenocytes may encounter membrane-free pRBC at a rate dependent on overall parasite burden. Potentially, splenic DCs may encounter very high ratios of membrane-free pRBC in high parasitaemia

settings, and as DC responses are not significantly affected by pRBC membrane, it is possible that the patterns reported in our study reflect physiological phenomena occurring in lymphoid organs.

An important point revealed in this work was that the DC response to frozen and thawed pRBCs is distinct from the response to fresh pRBCs (**Figure 3**). Methods to standardize frozen pRBC preparations have been used to prepare parasite vaccinations for standardization of controlled human malaria infection trials in naïve individuals (36). While this method induces viable parasites, it is likely that a proportion of ring-stage trophozoites would not survive the freezing process and may lead to induction of sub-optimal DC responses or tolerance. Furthermore, it serves as a caution for *in vitro* experiments that may use cryopreserved pRBCs as an immune stimulus. The data shown would argue that a comparison with freshly isolated pRBCs is needed to determine whether responses of human DCs and other innate responses *in vivo* and *in vitro* are qualitatively different when comparing inoculation with freeze-thawed vs. fresh pRBC preparations.

The surface activation marker most consistently suppressed by the presence of pRBCs, in both cDCs and pDCs, was CD40. Ligation of CD40 is crucial as it improves DC survival and enhances antigen presentation, therefore acting as a direct marker for DC activation (3). The interaction of CD40:CD40L between DCs and T cells activates both cell types and provides a vital positive feedback loop for cytokine secretion and co-stimulatory marker upregulation by DCs (3). Loss of CD40 on the surface of pRBC-exposed DCs could result in failure to induce downstream co-stimulatory pathways, resulting in overall failure of DC function. Work by Mukherjee and Chauhan highlights the importance of CD40 signaling for upregulation of other co-stimulatory markers in the moDC:*P. falciparum* context. When CD40L was added to moDCs co-incubated with "suppressive" doses of pRBCs, expression of CD83 was unaltered and expression of CD80 enhanced, rather than suppressed (51). A similar mechanism may be present in *bona fide* DCs. If CD40 expression is restored in DCs exposed to high doses of *P. falciparum*, it may restore their ability to stimulate T and B lymphocytes, which has implications for the design of next generation vaccine adjuvants that either target pRBC mechanisms of CD40 down-regulation or independently lead to enhanced expression of CD40 on DCs.

Murine FL-DC were used as a model for human blood DCs as they could be easily generated in large numbers and consistently produce similar levels of pDCs and cDCs (**Supplementary Figure 4**). FL-DCs also show greater transcriptomic similarities to their human counterparts than moDCs, another model commonly used to study malaria-DC interactions (9). The major difference between murine FL-DCs and human blood DCs is that only pDCs in humans express TLR9 (52). However, findings in human moDCs, which also do not express TLR9, are also suppressed by high doses of pRBCS (42, 53), suggesting high pRBC doses can suppress DC activation by multiple alternate pathways.

The mechanism by which pRBCs inhibit DC activation remains unclear. The effects of hemozoin upon DCs have been debated in the literature (18, 19) but it is unlikely to be the suppressive factor in this model as merozoite preparations were not hemozoin-depleted prior to DC co-incubation, yet still induced potent DC activation. A possible explanation may be found in the proteomic and transcriptomic differences between pRBCs and merozoites. The merozoite transcriptome is 45% smaller than that of the trophozoite. Merozoites lack the expression of many proteins required for hemoglobin digestion, protein export, and parasite metabolism (46, 47). One of these trophozoite-restricted protein families may contain the factor responsible for suppression; alternately, it may be a lipid, RNA byproduct or metabolite of the pigmented trophozoite, which is a very metabolically active life stage.

In conclusion, we demonstrate that pRBCs can suppress specific key DC functions and activation, in contrast to the activatory effect of merozoites. While DCs would interact with both merozoites and pRBCs in the blood, the specific suppressive effect of pRBCs appeared dominant and occurred even in the presence of merozoites. These findings may help partly explain why malaria immunity is typically slow to be acquired through natural exposure, why vaccine efficacy and vaccine-induced immune responses are lower in malaria endemic settings than among malaria-naïve subjects (54), and why immunity to malaria is generally not well-maintained (55). Further research to understand the mechanistic basis for these effects and identifying strategies to overcome them may be crucial for the development of highly efficacious and long-lasting vaccines.

ETHICS STATEMENT

This study was carried out in accordance with NHMRC guidelines and approved by the Animal Ethics Committee at AMREP Animal Services Precinct Animal Centre, Melbourne, Australia and Monash University, Clayton, Australia.

AUTHOR CONTRIBUTIONS

XZY and RJL carried out the research and analyzed the data. GF carried out essential experiments. JP contributed essential technical expertise. MOK and JGB designed and supervised the research and analyzed the data. XZY wrote the first draft. All authors contributed to writing of the manuscript.

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The Rough Guide to Monocytes in Malaria Infection

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While half of the world's population is at risk of malaria, the most vulnerable are still children under five, pregnant women and returning travelers. Anopheles mosquitoes transmit malaria parasites to the human host; but how *Plasmodium* interact with the innate immune system remains largely unexplored. The most recent advances prove that monocytes are a key component to control parasite burden and to protect host from disease. Monocytes' protective roles include phagocytosis, cytokine production and antigen presentation. However, monocytes can be involved in pathogenesis and drive inflammation and sequestration of infected red blood cells in organs such as the brain, placenta or lungs by secreting cytokines that upregulate expression of endothelial adhesion receptors. *Plasmodium* DNA, hemozoin or extracellular vesicles can impair the function of monocytes. With time, reinfections with *Plasmodium* change the relative proportion of monocyte subsets and their physical properties. These changes relate to clinical outcomes and might constitute informative biomarkers of immunity. More importantly, at the molecular level, transcriptional, metabolic or epigenetic changes can "prime" monocytes to alter their responses in future encounters with *Plasmodium*. This mechanism, known as trained immunity, challenges the traditional view of monocytes as a component of the immune system that lacks memory. Overall, this rough guide serves as an update reviewing the advances made during the past 5 years on understanding the role of monocytes in innate immunity to malaria.

Keywords: leukocytes, innate immunity, plasmodium, trained immunity, cytokine, phagocytosis

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INTRODUCTION

Malaria is a parasitic disease mostly present in poor tropical and subtropical countries. In 2016 alone, malaria accounted for 445,000 deaths and 216 million clinical episodes (1). When an infected female Anopheles mosquito feeds on human blood, she injects sporozoites, motile forms of the *Plasmodium* parasite, that travel to the liver. Within the hepatic cells, parasites divide to form schizonts that rupture and release merozoites into the bloodstream where they infect erythrocytes. The cycle of parasite division and merozoite invasion of new RBCs coincides with the clinical symptoms of malaria illness, which include fever, chills and headaches. The clinical symptoms progress from asymptomatic infection to uncomplicated disease to severe malaria to death. Life-threatening malaria occurs when infection leads to dysfunction of organs including the brain, placenta, kidney or lungs, or causes abnormalities in the patient's blood or metabolism, such as anemia.

Since sporozoites (the infective form) rapidly leave the skin (2), little is known about how skin innate immune cells interact with them. During blood stage infection, monocytes control parasite burden and contribute to host protection through phagocytosis, cytokine production and antigen presentation, but they also drive inflammation and sequestration of infected red blood cells (iRBCs) in organs (such as the brain, placenta, or lungs). Monocytes come in different "flavors" [discussed in (3)]. According to the levels of CD14 and CD16 expressed on their surface, they are classified in three subsets: classical or inflammatory ($CD14^{++}$ $CD16^{-}$), non-classical or patrolling ($CD14^{+}$ $CD16^{++}$) and intermediate ($CD14^{++}$ $CD16^{+}$). Classical monocytes, the largest subset, express the chemokine receptor CCR2, which mediates recruitment to sites of inflammation, where monocytes can differentiate *in situ* to macrophages or dendritic cell populations. Non-classical monocytes "patrol" the blood vessels to remove damaged cells and debris and resolve inflammation in damaged tissues [reviewed in (4)]. In mice, subsets are identified by Ly6C and CD11 markers (implicated adhesive interactions). $Ly6C^{hi}$ monocytes resemble the classical and intermediate human monocytes, and $Ly6C^{low}$ monocytes are similar to human non-classical monocytes. Human and mouse monocyte subsets play similar roles in host defense (5). In this rough guide, we summarize important recent discoveries related to the role of monocytes in innate immunity to malaria. For a summary of older literature, the reader is referred to Chua et al. (6).

ROLES OF MONOCYTES

Phagocytosis

Monocytes appear not to phagocytose RBCs infected with mature gametocytes, the sexual erythrocytic stage that transmits to the mosquito (7), but their ability to phagocytose merozoites and asexual iRBCs is pivotal to control of parasitemia (**Figure 1**). Antibodies are not essential for phagocytosis, but *Plasmodium*-specific IgGs enhance the phagocytic activity of monocytes and this correlates with protection and reduces the risk of symptomatic malaria (8–12). On the other hand, *Plasmodium* -specific IgEs and activated monocytes have a role in disease severity (11). The intermediate $CD14^{++}$ $CD16^{+}$ monocytes were the most efficient subset at phagocytosis of *Plasmodium vivax* iRBCs (which correlated with their expression of the adhesion molecules ICAM-1 and PECAM-1) (13) and IgG or complement opsonised *P. falciparum* iRBCs (10). With increasing age and malaria exposure, individuals develop protective IgGs to surface antigens of iRBCs, particularly to *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (8). Antibodies to merozoite surface proteins (MSPs) correlate with protection too. Opsonizing antibodies against MSP1 can recruit monocytes for merozoite phagocytosis (14), while cytophilic immunoglobulins (IgG1 and IgG3) against MSP2 and MSP3 strongly activate monocytes (15). Bergmann-Leitner reported that the relative phagocytic activity of monocytes *in vitro* (defined as "opsonization index") serves as a surrogate marker of protection induced by the RTS,S/AS01 vaccine. Surprisingly, they found that protected subjects showed lower opsonization efficiency (16). Likewise, non-opsonic phagocytosis, which largely relies on scavenger receptor CD36 (17), plays a role in removal of iRBCs, and might be particularly relevant in conditions in which antibody responses are compromised such as HIV infection. HIV infection additionally impairs monocyte functions, including non-opsonic phagocytosis of iRBCs (18). Malaria parasites also modulate monocyte protein expression. For example, iRBCs inhibit monocyte surface expression of complement receptor 1 (CR1 or CD35), and thus impair phagocytosis of circulating immune complexes that can bind to active C3b and C4b, potentially contributing to inflammatory pathology in malaria (19). Additionally the T-cell immunoglobulin- and mucin-domain-containing molecule 3 (Tim-3) that inhibits phagocytosis is down-regulated in monocytes during malaria infection (20).

Antibody-Dependent Cellular Inhibition

Antibody-dependent cellular inhibition (ADCI) is a major mechanism of defense in acquired immunity to malaria (21), in which monocytes, upon exposure to merozoites that have been opsonized with cytophilic antibodies [IgG1 and IgG3] subtypes to merozoite surface antigens (22), release soluble mediators that inhibit the growth of parasites in iRBCs (21, 23) (**Figure 1**). ADCI assay performed *in vitro* correlates with clinical protection from malaria and has been proposed as a functional assay for malaria vaccine studies (24). It has been used to assess the potential of merozoite antigens as vaccine candidates (25).

Cytokine Secretion

Following infection with *Plasmodium*, early secretion of pro-inflammatory cytokines by monocyte lineage cells helps to control parasite multiplication and resolution of infection, but excessive production contributes to pathogenesis. In humans, monocytes are an important source of these early cytokine responses (**Figure 1**).

Differences in activation and persistence of monocytic lineage cells between symptomatic and asymptomatic infection were reported in Haitian adults and might be due to higher production of GM-CSF, MIP-1β, or IL-34 upon exposure to *P. falciparum* schizont lysate from the PBMCs of these groups. These cytokines are likely drivers of a non-sterilizing immunity with lower parasite loads (premunition), by attracting and potentiating viability, opsonic phagocytosis and cytokine secretion in monocytes (26). In acute uncomplicated *P. falciparum* malaria in children, monocytes increase secretion of the proinflammatory cytokines TNF-α, IP-10 (CXCL10), IFN-γ, and IL-6 and decrease phagocytosis of iRBCs (27). In acute *P. vivax* infection, inflammatory mediators, TNF-α, IL-6, and IL-8, are primarily secreted by inflammatory and classical monocytes (28). TNF-α and IFN-γ influence the sequestration of iRBCs and activation of the endothelia by upregulating ICAM-1 and other adhesion molecules (28).

During blood-stage infection with *P. falciparum*, human inflammatory monocytes from naïve adults upregulate the expression of activation markers HLA-DR and CD86, which are associated with priming of T cells (29). In severe malaria in Malawian children, the inflammatory monocyte

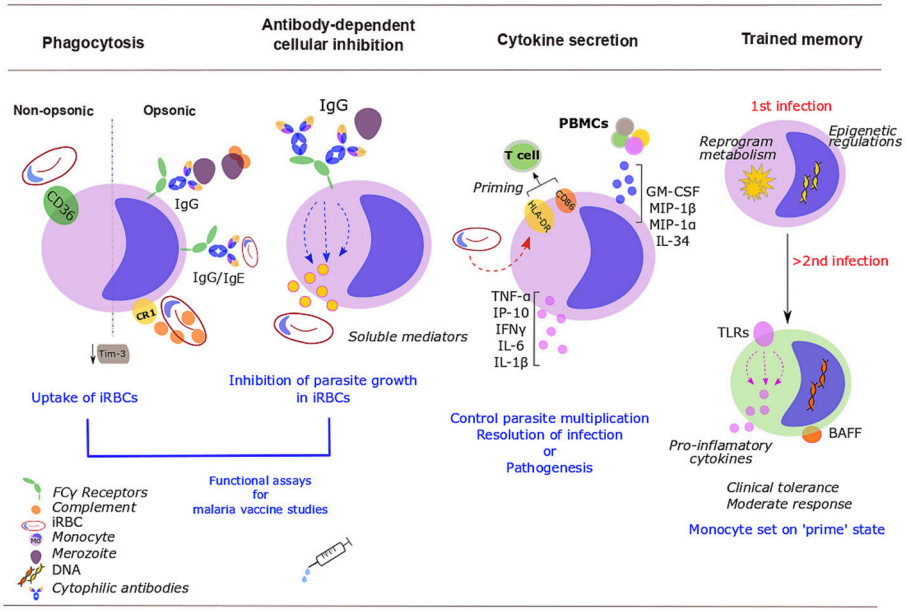

FIGURE 1 | Roles of monocytes during human malaria infection. Monocytes control parasite burden and contribute to host protection (or pathogenesis) through several mechanisms. Infected red blood cells (iRBCs) and merozoites are removed via opsonic or non-opsonic phagocytosis. Opsonic phagocytosis is mediated by either complement [binds to complement receptor 1 (CR1)] or malaria-specific antibodies (bind to Fcγ-receptors). Non-opsonic phagocytosis largely relies on CD36. Malaria down-regulates mucin-domain-containing molecule 3 (Tim-3). Soluble mediators released upon exposure to cytophilic antibodies stop *Plasmodium* from growing inside iRBCs [antibody-dependent cellular inhibition (ADCI)]. Monocyte phagocytosis and ADCI correlate with protection and might be used *in vitro* in malaria vaccine studies. Cytokine production balances protection/ susceptibility in the host. *Plasmodium* iRBCs increase HLA-DR; expression of activation markers, HLA-DR and CD86, might prime T cells response. PBMCs further activate and recruit monocytes through increased production of GM-CSF, MIP-1β, IL-34, TNF-α, or MIP-1α. Upon stimulation with *P. falciparum* iRBCs, monocytes secrete TNF-α, IL-1β, IL-6, IP-10, and IFN- γ. After a challenge with *Plasmodium,* monocytes develop some sort of memory. Monocytes undergo epigenetic modifications, metabolic rewiring and altered cytokine secretion. These changes "prime" monocytes to a more moderate response to secondary encounters with the parasite. Some of these changes will persist over time, including the expression of Toll-like receptors (TLRs) (involved in inflammatory cytokine production) and the membrane-bound form of the B-cell activating factor (BAFF).

subset was expanded and activated with higher plasma levels of inflammatory cytokines (IFNα, IFNγ, TNF-α, IL-6) and chemokines (CCL2, CCL3, CCL4, CXCL10) than in convalescence (30). In another study from Malawi, monocytes from children with severe malaria had lowered expression of the activation markers CD18, HLA-DR, and CD86. compared to healthy controls (31). When whole blood was stimulated *in vitro* with LPS, monocytes from children with severe malaria produced less proinflammatory cytokines TNF-α and IL-6 than cells from healthy controls. Exposure to *P. falciparum* iRBCs also alters monocyte activation. It resulted in upregulation of HLA-DR expression on naïve monocytes derived from haematopoietic stem cells (32). Monocytes from PBMCs of Papua New Guinean children with severe malaria responded *in vitro* to *P. falciparum* iRBCs by secreting higher quantities of TNF-α, MIP-1β, and MIP-1α (implicated in monocyte activation and recruitment) than healthy children or children with uncomplicated malaria (33).

SPLEEN

During the asexual stage, *P. falciparum* iRBCs become more rigid, and are retained by mechanical filtration in the spleen (34), which has a pivotal role in the immune response against malaria infection (35), reduction of parasitemia and clearance of infection (36).

In mice, upon infection with *P. chabaudi,* monocytes egress from the bone marrow and migrate to the spleen, reducing blood stage parasitemia by phagocytosing iRBCs and producing reactive oxygen intermediates (37). In deceased Malawian children, dysfunction in the ability of the spleen to phagocytose parasites has been linked with higher parasite loads and a more rapid progression to death (36).

Although the spleen does not contribute to the pool of circulating monocytes during *P. falciparum* infection in non-human primates (38), local splenic inflammatory monocytes could play various roles; *in situ*, murine splenic monocytes/macrophages stimulated by IFN regulatory factor 3 (IRF3) (39) coach activated $CD4^+$ T cells toward a protective Th1 fate during infection with blood-stage *Plasmodium* parasites (39, 40). Murine splenic monocytes also migrate to the brain as $CCR5^+CXCL9/10^+$ MO-DCs inducing neuroinflammation (41). Little work has been done on human spleen; differences and similarities in the pathological changes observed in the spleens of human and mice during *Plasmodium* infection are discussed in Urban et al. (42).

ROLE OF MONOCYTES IN CLINICAL MANIFESTATIONS

Severe Malaria Anemia

Severe malaria anemia (SMA) [hemoglobin <5.0 g/dL], is the most common severe manifestation of malaria in young children and pregnant women. SMA is caused by sequestration of RBCs in the spleen, loss of both RBCs and iRBCs and suppression of erythropoiesis and abnormal development of RBCs precursors (dyserythropoiesis) in the bone marrow (43). During SMA, monocytes contribute to SMA through phagocytosis and secretion of pro-inflammatory cytokines (43). Past studies found a correlation between monocytes loaded with *P. falciparum* hemozoin (a parasite by-product) and suppression of erythropoiesis; while high levels of these monocytes predicted SMA (44). More recent studies in non-human primate macaque models for *P. vivax* infection showed that during acute malaria, monocytes loaded with hemozoin suppress erythropoiesis in the bone marrow by inducing apoptosis of the erythroid progenitors via IFNγ and antagonization of GATA1 transcriptional networks (45).

Cerebral Malaria

Cerebral malaria (CM) is the most life-threatening presentation of *P. falciparum* malaria in young African children. Impaired consciousness, delirium or coma may be accompanied with swelling of the brain, intracranial hypertension or changes in the retina (46). Sequestered iRBCs block intracerebral blood vessels, and infiltrating immune cells including monocytes accumulate in the same vessels and secrete inflammatory cytokines (47); monocyte accumulation is greater in HIV-infected CM children (48).

During experimental cerebral malaria (ECM) in *P. berghei* ANKA-infected mice, $Ly6C^{hi}$ monocytes are the main sequestered leukocyte population, inducing inflammation and disease (49), and aggravating brain inflammation by recruiting $CD4^+$ and $CD8^+$ T cells (49, 50) and by secreting the chemokine CXCL10 (50, 51). CXCL10 mediates cerebral adhesion and accumulation of T cells, driving the onset of CM (51). In *P. berghei* ANKA-infected mice also, *Plasmodium* specific $CD8^+$ T cells regulate the adhesion and rolling behavior of monocytes (49). Other molecules that might mediate monocyte accumulation in *P. berghei* ANKA model include increased C5a (28) and inhibition of nitric oxide activity (52) and the chemokine MCP-1/CCL2 (53).

At present, there is no clear consensus on the extent to which findings in mice models translate to humans, given data suggesting that *P. berghei* ECM is primarily driven by leukocyte accumulation, whereas human CM is principally due to iRBC sequestration in the cerebral vasculature (54).

Placental Malaria

Malaria in pregnant women can restrict fetal growth or result in premature delivery and elevates the risk of maternal anemia and infant mortality. Infection is initiated by the sequestration of iRBCs that bind to the chondroitin sulfate A expressed on the placenta through VAR2CSA, a member of the PfEMP1 protein family (55, 56). Subsequently, circulating monocytes and tissue-resident macrophages accumulate in the intervillous space of *P. falciparum*-infected placentas initiating a local inflammation (intervillositis), a major determinant of the severity of the disease and intrauterine growth restriction (57).

Recently, Aubouy et al. described a protective role for monocytes and macrophages during pregnancy (58). The expression of the scavenger receptor CD36 and Heme-Oxygenase-1 (HO-1) on circulating monocytes correlated with the levels of anti-inflammatory markers IL-10 and CD163, and with an increase in infant birth weight (58). These findings diverge from other works that link high levels of IL-10 in both plasma and placenta with low birth weight babies and parasitemia (59). In addition, high numbers of circulating monocytes and high plasma IL-10 concentrations predict maternal anemia at delivery (60) although how these two factors entangle is unknown. The membrane receptor CD163 contributes to the anti-inflammatory response by scavenging hemoglobin: haptoglobin complexes, resolving monocyte activation and improving clinical outcome (58). Levels of soluble sCD163 shed by monocytes correlate negatively with birth weight and maternal hemoglobin levels (61).

Monocyte opsonic phagocytosis of iRBCs is also an important component of the acquired immune response in pregnancy malaria. Multigravid women generate protective IgG antibodies to VAR2CSA, (9) and antibodies recognizing domains DBL5 and DBL3 are effective inductors of monocyte phagocytosis (12). Limited cross-reactivity between isolates may hinder the development of a vaccine (12).

Acute Lung Injury

Children and adults experiencing severe malaria are prone to develop acute lung injury (ALI) and its most severe form, acute respiratory distress (ARDS). Patients with ARDS display dyspnoea, cough, and chest tightness and can develop hypoxia leading to death. These complications arise probably from increased alveolar capillary permeability, triggered by iRBC sequestration in pulmonary vasculature and secondary local inflammation followed by cytokine secretion. Bronchial IL-33 might be a driver of pulmonary edema in human patients since it positively correlates with $CD68^+$ monocyte accumulation (62).

In mice, most leukocytes in the pulmonary interstitium are bone-marrow derived inflammatory monocytes (63) and macrophages (64). In this model, although monocytes prevent tissue damage by CD36-mediated non-opsonic phagocytosis of iRBCs (63), they might also contribute to the inflammatory manifestations of ARDS. The β_2 leukocyte integrin, $\alpha_D\beta_2$ (CD11d/CD18) increases alveolar-capillary membrane permeability, the accumulation of monocyte and macrophages, and lung edema (64), while another β_2 integrin, $\alpha_M\beta_2$ (CD11b/CD18) is important for parasite clearance during ALI (65). In humans, post-mortem histology reveals monocyte accumulation in pulmonary vessels (47), which could also explain the impaired gas transfer observed in adults with uncomplicated malaria (66).

In summary, severe forms of malaria are linked to the ability of iRBCs to sequester in the vasculature of organs like

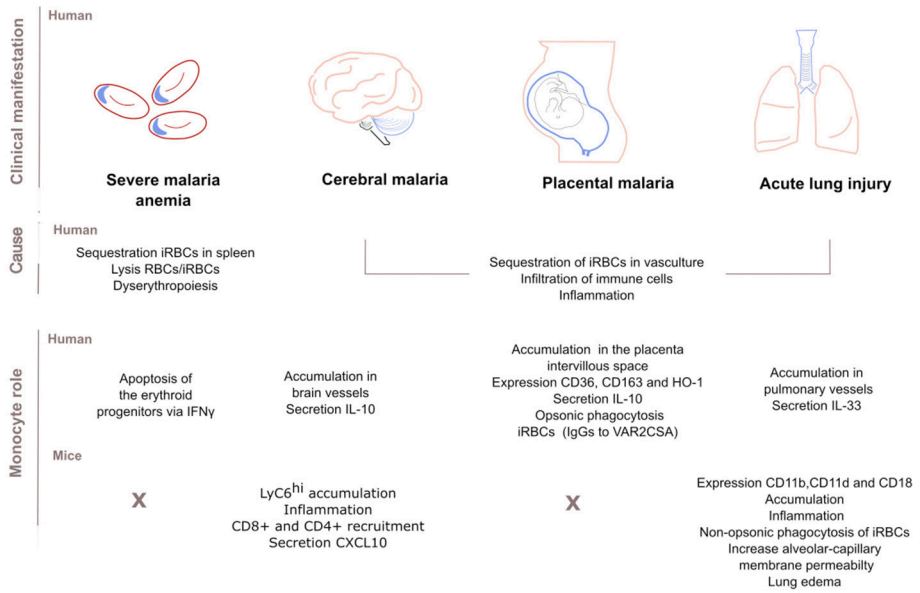

FIGURE 2 | Role of monocytes in the clinical manifestations of malaria. Sequestration of infected red blood cells (iRBCs) in the vasculature of main organs (spleen, brain, placenta, or lungs) is associated with severe disease. Infiltration of immune cells and subsequent inflammation also contributes to pathogenesis. Dyserythropoiesis and lysis of uninfected RBCs and iRBCs cause severe malaria anemia too. Human monocytes infiltrate and accumulate in the vessels of those main organs. There, monocytes secrete anti- or pro-inflammatory cytokines, express surface markers or phagocytose IgG-opsonised iRBCs. Studies disagree over whether these mechanisms drive host protection or susceptibility. Similarly, mouse monocytes accumulate and drive inflammation in the brain and lung. In the mouse model of cerebral malaria, monocytes further recruit $CD8^+$ and $CD4^+$ cells by secreting the chemokine CXCL10. In the lung, monocyte CD11b/CD18 expression is important for parasite clearance while the integrin CD11d/CD18 expression increases permeability of the alveolar-capillary and causes lung edema. There is no clear consensus on to what extent findings in mice models of malaria infection translate to humans but human and mouse monocyte subsets play similar roles in host defense.

lungs, placenta, brain, or spleen. After iRBC sequestration, immune cells including monocytes may accumulate in the vasculature. Local monocytes become an immune hub by removing merozoites or iRBCs, inhibiting parasite growth, secreting cytokines or recruiting other cells of the immune system; such activities might improve or worsen the progress of clinical manifestations. Some of the roles of monocytes in severe disease are illustrated in **Figure 2**.

EFFECT OF MALARIA PARASITES ON MONOCYTE FUNCTIONS

Beyond the effect of iRBCs sequestration and lysis, hemozoin, parasite DNA or secreted vesicles also contribute to malaria pathogenesis. *Plasmodium* digests hemoglobin within RBCs. The metabolic by-product, heme, is polymerised into the crystal structure hemozoin (HZ) (67). Circulating and resident monocytes phagocytose and accumulate HZ. The proportion of circulating HZ-containing monocytes increases during malaria (31) and this correlates with disease severity (68). Particularly, HZ-containing monocytes are significantly elevated in patients with SMA (69), and HZ appears to be important in the induction of dyserythropoiesis and apoptosis in nascent erythroid cells (69). Overall, ingested HZ weakens the immune system by destroying monocytes (70), impeding their maturation to dendritic cells (71) or impairing their overall functionality (72).

HZ influences monocyte function in a number of ways. It exacerbates the production of pro-inflammatory markers such as IL-1β and TNF-α (73). This may be due to the association between HZ crystals and the lipid 15-HETE (15-hydroxy-eicosatetraenoic acid), which upregulates expression and release of matrix-metalloproteinase 9 (MMP-9), in turn implicated in the secretion of inflammatory cytokines (74). HZ also induces monocyte dependent expression and secretion of TIMP-1, the endogenous inhibitor of MMP-9 (75), although the role of TIMP-1 in malaria pathogenesis remains unknown. HZ lowers monocyte expression of adhesion molecules (CD11b, CD11c, and CD18) (31), and diminishes *in vitro* monocyte diapedesis and chemotactic motility toward MCP-1, TNF-α, and FMLP (formyl-methionyl-leucyl-phenylalanine), partially explaining patients' immunosuppression (31). In mice, pulmonary HZ is associated with the recruitment of inflammatory cells, including inflammatory monocytes (76).

From within RBCs, *Plasmodium* communicates with other cells by releasing vesicles to the extracellular milieu. Monocytes internalize these extracellular vesicles (EVs) (77). EVs from erythrocytes infected with ring-stages of *P. falciparum* modify the functionality of human monocytes, in part by upregulating antigen presentation pathways and enhancing the interferon response (78), although EVs containing PfEMP1 downregulate "defense response" pathways (78), consistent with observations that PfEMP1 suppresses the immune response by dampening monocyte inflammatory cytokine and chemokine release (79). Vesicles may also deliver non-coding parasite RNAs and gDNA

to the monocyte (77). Once inside, specific cytosolic sensors detect *Plasmodium* DNA, triggering the transcription of type I IFN genes by the stimulator of TNF genes (STING) pathway (77). Interestingly, the ingestion of circulating DNA-containing immunocomplexes (ICs) is an alternative way for parasite's DNA to gain access to the monocyte cytosol (80). In this case, ICs induce the assembly of the NLRP3/ASC$^+$ and AIM2/ASC$^+$ inflammasomes, activation of caspase-1 and secretion of IL-1β (80). In parallel, DNA bound to HZ leads also to caspase-1 dependent IL-1β secretion but through NLRP12 and NLRP3 inflammasome (81). More importantly, inflammasome assembly induces a "primed" state in monocytes which is partially dependent on TLR9 activation, and when exposed to a second microbial challenge these cells produce deleterious amounts of IL-1β (81).

EFFECT OF MALARIA PARASITES ON CIRCULATING MONOCYTE COUNTS

As the disease progresses, malaria alters blood cell counts. This might correlate with the ability of the individual to mount a proper immune response and reflect different levels of immunity to malaria (82). As such, changes in leukocyte numbers and cytokine profiles have been assessed as markers for the course of the infection and the immune response (83). In malaria- naïve volunteers, during the liver stage of *P. falciparum*, neutrophil, lymphocyte and monocyte counts increase (84). This is consistent with other studies reporting an increase in CD14$^+$ cells in primary *P. falciparum* infection (20) and an expansion of the inflammatory intermediate CD14^{++}CD16$^+$ monocyte subset during uncomplicated *P. falciparum* malaria in children (27). In contrast, both increased (13) or decreased (85) numbers of circulating monocytes have been observed in patients during *P. vivax* infection.

Leukocyte ratios might constitute surrogate markers for immunity. The monocyte to neutrophil ratio has been associated with severe malaria, especially in semi-immune patients (82). If low, this ratio may indicate a risk for developing complicated malaria (86). By contrast, a high monocyte to lymphocyte ratio (MLCR) better discriminates between clinical malaria and controls (82), correlating with increased risk for clinical malaria (87). Most importantly, variation in RTS,S vaccine efficacy between individuals is significantly predicted by differences in the MLCR ratio (88).

Some studies report associations between high circulating monocyte counts and high parasitemia (85), but others report that monocyte counts are significantly lower in patients with high parasitemia (89). This disagreement might be due to differences in the hematological profile of circulating cells between geographical areas (90). Regardless of the number of malaria episodes experienced, age and season also affect hematological indices and white blood cell subsets, including the monocyte count (91). Still, it is possible to establish reference intervals for hematological parameters that are comparable and applicable across areas with similar transmission conditions (92). Leukocytes also undergo changes in volume, conductivity and light scatter that reflect changes in function in different types of infections (93). In clinical malaria, monocytes increase their volume and relative quantity (94) while their internal composition (conductivity) significantly differs from that observed in non-malaria fevers (93).

TRAINED IMMUNITY IN MONOCYTES

Vaccines target the adaptive response, but clinical and epidemiological data prove that vaccines such as BCG exert nonspecific effects too (95). Possible mechanisms included "heterologous immunity," driven by cross-reactive T-lymphocytes; or trained memory in innate immune cells. After a first stimulus, the "trained immunity" phenotype relates to a "prime" state that enhances reactivity of monocytes/macrophages or NK cells to a secondary challenge (96). This phenotype involves epigenetic modifications, metabolic rewiring or cytokine secretion. This may well be important in malaria, where each new infection with *Plasmodium* activates the innate response (27).

Reinfections with *Plasmodium* can alter monocyte metabolism, chromatin, receptors expressed or the frequencies of each subset. But these alterations may either lower (tolerance) or increase host resistance (trained immunity) to reinfections (75) (**Figure 1**).

Compared to vivax-naïve individuals, semi-immune people reprogram their myeloid cells' metabolism to a more coordinated response which could influence clinical tolerance to reinfections and result in asymptomatic infections with *P. vivax* (97). This acquired tolerance moderates the immune response to *P. vivax* infection, as observed in gene transcription profiles in peripheral blood comparing semi-immune to malaria-naïve individuals (98).

The largest nomadic ethnic group in Africa, the Fulani, are more resistant to *P. falciparum* than their geographical counterparts (99). Global transcriptional and DNA methylation analysis of the whole blood show that the chromatin in Fulani people's monocytes (and no other cell) is set on a prime state; thus upon *P. falciparum* infection, epigenetic regulations in monocytes induce an enhanced pro-inflammatory response. Compared to a sympatric group, Fulani adults show higher levels of inflammasome activation, and in the presence of malaria infection this translated into higher secretion of IL-1β and IL-18 (99).

It is thought that pattern recognition receptors including TLRs could assist monocytes to mount some sort of memory specific to individual organisms, including *Plasmodium* (100). Children with severe malaria show a lowered expression of TLR2 and TLR4 which correlates with monocyte inactivation and reduced inflammatory cytokine production (31). In the acute phase of infection, monocytes overexpress genes involved in TLR signaling (TLR8, LY96, MYD88) (27). More importantly, these changes persist in convalescence when compared to monocytes from healthy malaria-naive controls (27). Another long-lasting effect is the increased expression on various monocyte subsets of the membrane-bound form of B-cell activating factor (BAFF), essential in B-cell homeostasis (101).

History of exposure also influences the relative number of circulating monocyte sub-populations (102). In malaria-naive individuals, frequencies of classical and intermediate monocyte sub-populations expand during blood stage infection with *P. falciparum* (29). In Kenyan children, after recovery from acute uncomplicated *P. falciparum* malaria, the inflammatory "intermediate" subset stops its expansion and returns to levels of healthy asymptomatic children (27).

FUTURE DIRECTIONS

Caution must be taken when interpreting data regarding monocytes' roles in malaria infection. Monocytes are an heterogenous population of cells whose functionality is further shaped by host age, geography and history of exposure (31). This is consistent with training or tolerance effects that could explain the *contradictory behavior* of monocytes observed across different settings, independent of differences in protocols and analysis. To minimize these discrepancies, Udomsangpetch et al. have developed a model of mononuclear cells generated from hematopoietic stem cells, that evaluates *in vitro* the interaction between naïve immune cells and malaria parasites (32). Whole genome association studies (GWAS) might be used as a tool to identify genetic differences that can further explain why monocytes respond differently across geographical areas (103, 104). Other inconsistencies, like responses to malaria vaccines reported to date, may in part be attributed to variations in the monocyte response (75), influenced by the adjuvant used and the age of the patient (105).

As we saw in this *rough guide,* the extraction of monocytes from peripheral blood is a common method to study their response in isolation. However, it will be important to consider analyses that integrate third players in the interaction between monocytes and parasites. Gene expression profiling of whole blood might be used to identify the type and duration of the immune response in infection (98). But as Zak et al. point out, innate responses in the periphery might not reflect what happens locally: monocyte re-localization to an inflammatory site could explain why a gene is less present or transcribed in blood (106). In this regard, systems vaccinology offers a powerful approach quantifying innate and adaptive responses in different compartments (106).

AUTHOR CONTRIBUTIONS

AO-P gathered all the papers included in the review, drafted the manuscript and designed (**Figures 1**, **2**). SR corrected and revised it critically for important intellectual content, SR also gave final approval for the version submitted.

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REVIEW
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Neutrophils and Malaria

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Neutrophils are abundant in the circulation and are one of the immune system's first lines of defense against infection. There has been substantial work carried out investigating the role of neutrophils in malaria and it is clear that during infection neutrophils are activated and are capable of clearing malaria parasites by a number of mechanisms. This review focuses on neutrophil responses to human malarias, summarizing evidence which helps us understand where neutrophils are, what they are doing, how they interact with parasites as well as their potential role in vaccine mediated immunity. We also outline future research priorities for these, the most abundant of leukocytes.

Keywords: neutrophil, malaria, *Plasmodium*, immunity, antibody mediated immunity, polymorphonuclear (PMN)

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INTRODUCTION

Neutrophils (also known as polymorphonuclear cells) are the most common white blood cell in the body (1). They can clear pathogens by phagocytosis; by producing reactive oxygen species (ROS) and other antimicrobial products; or by formation of neutrophil extracellular traps (NETs) (2). Additionally, they also play a role in the activation and regulation of the immune response, by cytokine and chemokine secretion (3), and possibly antigen presentation (4). Their importance in controlling infection can be highlighted by the increased susceptibility to fungal and bacterial infections seen in individuals suffering from neutrophil deficiencies (5). Malaria is a disease caused by infection with *Plasmodium spp.* parasites. It causes severe morbidity and mortality, and young children and pregnant women are especially susceptible to disease. Malaria is a large public health burden with an estimated 216 million cases of malaria being reported in 2016, resulting in an estimated 445,000 deaths (6). Globally most disease caused by infection with *Plasmodium spp.* is caused by *P. falciparum* (6). Pathology is thought to be due to a combination of the sequestration of infected red blood cells (iRBC) in the microvasculature, endothelial activation, as well as pro-coagulant and importantly pro-inflammatory responses (7). In this review, we assess the literature examining how neutrophils and *Plasmodium spp.* parasites interact, and the mechanisms by which neutrophils can play an active role in parasite clearance.

NEUTROPHIL DYNAMICS AND RECRUITMENT TO SITES OF PARASITE SEQUESTRATION

Changes in peripheral blood neutrophil levels have been described during *Plasmodium spp.* infections. In controlled human malaria infections (CHMI) in non-immune individuals, neutrophil numbers are stable during the asymptomatic liver stage (8). In naturally-infected individuals, patterns of change in peripheral blood neutrophil numbers vary with the cohort studied. Using hematological data from over 3,000 children, Olliaro et al. estimated that peripheral blood neutrophil counts increase about 43% (95% CI 26–35%) during acute uncomplicated malaria, and that the level of increase is positively associated with parasitaemia (9). In semi-immune travelers neutrophil counts were higher in those with severe malaria compared to those with uncomplicated malaria, while in non-immune travelers, though neutrophil counts increased with the presence

of infection, neutrophil counts did not vary with disease severity (10). A study in HIV-infected individuals showed no difference in neutrophil numbers when comparing those with and without asymptomatic *P. falciparum* infection (11), whereas pregnant women with *P. falciparum* infection had lower numbers of peripheral blood neutrophils than uninfected women (12). Differences between cohorts are likely due to disease status classification (clinical malaria or asymptomatic parasitemia), immune status and/or age.

Neutrophils are a heterogenous population and this is important because different neutrophil subsets can have varying functional properties, for example CD177+ neutrophils are also positive for Proteinase 3, and IL17+ neutrophils have increased ROS production [reviewed in (13)]. We know that neutrophils from individuals infected with *Plasmodium spp.* behave differently compared to those from non-infected individuals (14–18), and a subset of neutrophils with impaired oxidative burst have been observed in individuals infected with *P. falciparum* (18), suggesting that neutrophil subsets change during the course of infection. In individuals challenged with LPS, inflammation results in the release of a neutrophil subset that suppresses T cell activation (19), whether this occurs during *P. falciparum* infection is unclear but it is one example of why work to identify neutrophil subsets in *Plasmodium spp.* infections would likely yield valuable information into the role of neutrophils in malaria.

Neutrophils are generally the first circulating cells to respond to an invading pathogen. However, how and whether neutrophils are recruited to the sites of iRBC sequestration is still unclear. We know very little regarding neutrophil expression of receptors involved in migration and adhesion. There is no evidence that neutrophil adhesion molecule CD11a changes with infection (18), and expression by neutrophils of other adhesion molecules such as CD18, CD11b, and CD62L is still unstudied. There is more information on the expression of neutrophil receptors on endothelial cells. Expression of receptors on endothelial cells involved in neutrophil adhesion and migration are likely increased with infection. Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the endothelial leukocyte adhesion molecule E-selectin are increased on endothelial cells after exposure to iRBC *in vitro* [reviewed in (20)] and this is supported by observations showing increased levels of soluble E-selectin and soluble ICAM-1 in the blood of *P. falciparum* infected individuals (21). Regarding chemokines involved in neutrophil recruitment, neutrophil chemoattractant protein CXCL8 is increased in peripheral blood of patients with severe malaria [reviewed in (22)] (23) as well as in the cerebral spinal fluid (CSF) of children with cerebral malaria and in the placentas of women with malaria in pregnancy [reviewed in (22)]. In addition, *P. falciparum* antigen can induce the production of neutrophil recruitment chemokines CXCL1 and Interleukin 8 (IL8) production by endothelial cells and the production of Interleukin 8 (IL8) by placental syncytiotrophoblast [reviewed in (22)]. Interestingly, although increased expression of neutrophil chemoattractants occurs, studies of malaria pathology rarely show significant neutrophil infiltration at sites of sequestration.

Low numbers of neutrophils were reported in the brain microvasculature in autopsy samples from children in Malawi (14), and neutrophil numbers were not significantly higher in placentas infected with *P. falciparum* or *P. vivax* compared to non-infected placentas (24, 25). An exception is chronic *P. falciparum* placental malaria, which can be accompanied by massive intervillous inflammation, with increased numbers of CD15+ granulocytes (predominantly neutrophils) (26). When the lungs were studied, one study from Thailand showed no difference in neutrophil levels (measured by the presence of elastase positive cells) between fatal *P. falciparum* malaria with or without pulmonary oedema and controls who died from trauma (27). By contrast, in fatal *P. vivax* infection, interstitial lung infiltrates consisting of CD15+ cells were reported from Brazil. Caveats are that some individuals suffered co-pathologies and there was no non-infected control group for comparison (28).

The "snap shots" of pathogenesis provided by tissue samples, together with neutrophils' short half-life (29), mean that it is hard to exclude neutrophil recruitment to sites of infection, however the data to date suggest recruitment is not always happening; why is this? One possible answer is that *Plasmodium spp.* infection inhibits neutrophil chemotaxis. Neutrophils from individuals with symptomatic *P. falciparum* malaria have reduced chemotaxis compared to non-infected healthy controls (15) and this is restored 7 days after treatment, suggesting the involvement of parasite antigens. Neutrophils from individuals with cerebral malaria have reduced chemotaxis to Interleukin 8 (IL8) and N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) (14), and this reduction in chemotaxis may be partly due to increased free heme (14, 18) and/or decreased neutrophil expression of IL8 receptor CXCR2 (as reported in *P. vivax* malaria) (14, 30). Additionally, blood-stage parasite antigens inhibit proinflammatory protein S100-calcium binding protein (S100P) stimulated chemotaxis of the neutrophil cell line HL-60 *in vitro* (31, 32). Whether the reduced chemotaxis observed *ex vivo* explains the lack of neutrophil recruitment to the sites of parasite sequestration is still uncertain and warrants further investigation (20, 21, 33).

NEUTROPHILS AND PARASITE CLEARANCE: PHAGOCYTOSIS, ROS AND NETS

Neutrophil Phagocytosis of *Plasmodium spp.*

Phagocytosis is one way that neutrophils play a role in the clearance of malaria parasites. Neutrophils express immunoglobulin (Ig) binding receptors Fcγ receptor I (FcγRI) (after activation), FcγRII and FcγRIII as well as complement receptors complement receptor 1 (CR1) and CR3. Together these can facilitate phagocytic uptake of antigen opsonised with components such as IgG or C3b [reviewed in (34)] (**Figure 1A**). Neutrophils are known to phagocytose iRBC *in vivo* as observed in blood films from children (35) and in bone marrow aspirates which show phagocytosis of merozoites and occasionally

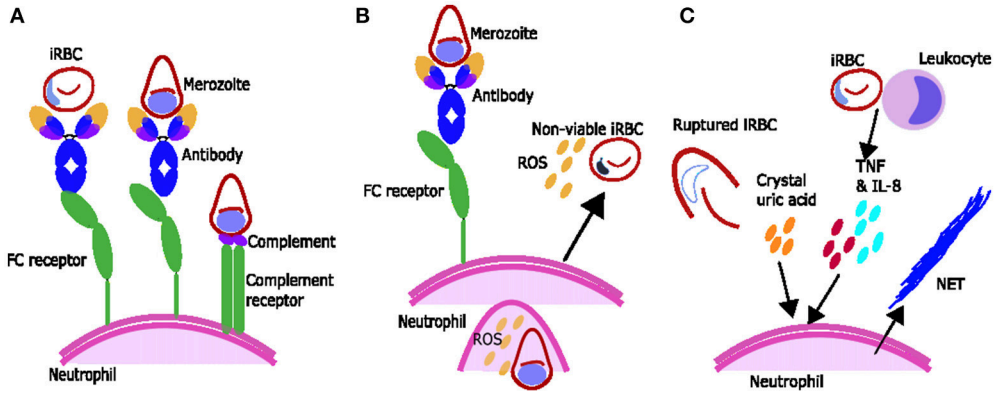

FIGURE 1 | Proposed mechanisms of parasite clearance by neutrophils during *Plasmodium spp.* infection. **(A)** Neutrophils can phagocytose blood stage parasites after opsonization with antibodies and possibly opsonization with complement. **(B)** Neutrophils exposed to merozoite antigen opsonized with antibody produce reactive oxygen species (ROS) which can inhibit intraerythrocytic parasite growth. **(C)** Components associated with *Plasmodium spp.* infection including inflammatory cytokines TNF and IL-8 produced by leukocytes and crystal uric acid released upon infected red blood cell (iRBC) rupture may induce neutrophils to produce neutrophil extracellular traps (NETs).

trophozoites by neutrophils and neutrophil metamyelocytes (neutrophil precursors undergoing granulopoiesis) (36).

Neutrophil phagocytosis of merozoites is influenced by several factors. Phagocytosis of merozoites opsonised with non-immune serum has been observed *in vitro* (37) and has possibly been observed *ex vivo* in the blood of ex-service men who had returned from Vietnam infected with *P. falciparum* (38). Neutrophil phagocytosis of merozoites opsonised with non-immune serum is possibly dependent on complement, as heat inactivation decreases phagocytosis, and can be increased if the neutrophils are pre-treated with factors such as tumor necrosis factor (TNF), interferon γ (IFNγ), IL1β or granulocyte-macrophage colony-stimulating factor (GM-CSF) (37). Antibody toward merozoite antigens also promotes phagocytosis, which is much higher if merozoites are opsonised with immune serum compared to non-immune serum (37).

Unlike phagocytosis of merozoites, which may involve complement, phagocytosis of iRBC is largely dependent on the presence of Ig. Neutrophils isolated from malaria infected children can phagocytose schizonts *in vitro* (16), but neutrophils from American service men infected with *P. falciparum* (38) (with limited previous exposure to *P. falciparum* and therefore little Ig toward the iRBC) did not. In addition, sera from individuals living in endemic areas promote phagocytosis of iRBC and this activity is dependent on IgG in the serum (39) and independent of complement (40, 41). It is not known if Ig mediated phagocytosis of parasites by neutrophils is associated with protection from disease.

Intra-erythrocytic gametocytes are not very susceptible to neutrophil phagocytosis (40). However, it is possible that neutrophil phagocytosis of extracellular gametes while in the mosquito gut could play a role in transmission-blocking immunity. Neutrophils phagocytose gametes *in vitro* in conditions similar to those of the mosquito gut when immune sera is present and this phagocytosis is dependent on gametocyte antibodies, especially IgG (40). *In vivo* it has been shown that neutrophils also phagocytose gametes inside mosquito midguts, and that this phagocytosis is enhanced by the presence of immune serum in the blood meal (40). However, although the presence of neutrophils and individual sera in the blood meal decreases infectivity (42), there is no evidence of an association between levels of neutrophil phagocytosis, promoted by serum *in vitro*, and the ability of that serum to affect mosquito infectivity in experimental settings (40).

In rat models, neutrophils phagocytose exoerythrocytic parasites (43), however *ex-vivo* evidence of a role of neutrophil phagocytosis and immunity toward this parasite stage in humans (both *in vitro* and *ex vivo*) is lacking.

Key unanswered questions include the importance of neutrophil phagocytosis in protection from infection or disease and in blocking of transmission. Studies examining associations between antibody induction of neutrophil phagocytosis of different parasite life cycle stages and patient outcomes could help determine the importance of neutrophil phagocytosis in protection from disease.

Reactive Oxygen Species and *Plasmodium spp.* Infection

Neutrophils can clear pathogens by respiratory burst, the conversion of oxygen to superoxide by nicotinamide adenine dinucleotide phosphate oxidase (NAPDH) oxidase (NOX). This superoxide is converted into hydrogen peroxide and hydroxyl radicals, together referred to as ROS. NOX is located on both the neutrophils plasma and phagosomal membranes and the ROS it produces can diffuse across membranes. This means that ROS are present in the phagosome, intracellular and also extracellular spaces [reviewed in (44)] and can therefore play a role in killing both phagocytosed intracellular as well as extracellular parasites.

ROS from activated neutrophils are capable of inhibiting parasite growth *in vitro*, and studies using various ROS inhibitors or scavengers suggest singlet oxygen, rather than hydrogen peroxide or superoxide, is responsible for this inhibition (45, 46). Growth inhibition by ROS occurs during the intra-erythrocytic development stage of the parasite (45, 46) (**Figure 1B**), rather than during merozoite invasion of erythrocytes, which is not inhibited by ROS production from stimulated neutrophils *in vitro* (47). *Ex vivo* data suggests that during malaria infection neutrophils are activated to produce ROS and that ROS may have a role in parasite clearance. Neutrophils from children with malaria inhibit parasite growth *in vitro* better than neutrophils from uninfected children or adults (16) and oxygen consumption is higher [indicating activation and production of ROS (48)] in neutrophils from people with symptomatic acute malaria compared to controls (17). In addition, children with faster parasite clearance times have neutrophils which produce more ROS (49).

ROS production by neutrophils is influenced by a number of factors, including the host's genetic background (50). In the presence of antigen neutrophils produce higher amounts of ROS with immune serum compared to non-immune serum (51) or compared to IgG depleted serum (52), supporting a role for IgG-Fc interactions in ROS production (52) (**Figure 1B**). Regarding complement, one study suggests serum heat inactivation reduces ROS production (51) and another showed that heat inactivation changes the dynamics of ROS production (53). However, the activation of neutrophils to produce ROS by heat-inactivated serum (52) and by purified IgG (52, 53) suggests that the full complement cascade is not necessary.

The ability of Ig to induce ROS has been called antibody dependent respiratory burst (ADRB), and assays measure different components of this process. In solid-phase assays (where the antigen is bound on a plate), ROS are secreted from the neutrophil and this process is largely dependent on FcγRIIa. By contrast, when neutrophils phagocytose whole merozoites the resulting ADRB and associated ROS production occur within the neutrophil are only partially dependent on FcγRIIa (53). Antibodies capable of inducing respiratory burst are acquired with exposure to *Plasmodium spp.* In a small cohort study, sera taken after an immune episode and incubated with merozoites induced more neutrophil ROS production than sera taken during the malaria episode, and sera from adults induced more neutrophil ROS production than sera from young children (54). ADRB toward merozoite antigens was higher in a holo-endemic area compared to a meso-endemic area (52) and increased with age (55), and antibodies capable of inducing respiratory burst have been associated with protection from malaria. Individuals whose serum induced a high ADRB toward merozoites were less likely to experience clinical malaria when compared to those whose serum induced a low ADRB (52, 55), and a combined measure of ability of an individual's serum sample to induce both ADRB and growth inhibition has been associated with protection from severe malaria (56). Supporting a role for ADRB in protection from disease, polymorphisms of FcγRIIIb [a neutrophil receptor that is involved in IgG dependent ROS production (57)] that improve neutrophil Fc-Ig binding are associated with protection from febrile malaria (58, 59).

Antibodies opsonising merozoites or merozoite antigens can induce ROS production by neutrophils, whereas there is no evidence that antibodies opsonising iRBCs result in a major ROS response (17, 51, 52, 54, 60). The reasons for this difference are unclear as IgG are known to recognize the surface of iRBC (61). The merozoite antigens which are targets for ADRB include *Plasmodium falciparum* merozoite surface protein-5 (PfMSP5), MSP1-19 and MSP1, as indicated by correlations between IgG antibodies to these antigens and ADRB (55, 62, 63), and by antigen/antibody depletion assays with MSP1 or its C terminal domain MSP1-19 (62, 63). See **Figure 1B** for diagram for ROS production.

Antibodies which induce ROS may be similar to those which promote merozoite phagocytosis, as the functions are correlated in neutrophils responding to opsonised merozoites (54). Antibody subclass may be important: mouse-human chimeric IgG toward MSP1-19 is sufficient to induce NADPH-mediated oxidative burst (and degranulation) from neutrophils, but mouse-human chimeric IgG3 toward the same antigen is not (64). Multiple antibody isotypes may be involved ROS production. Recombinant human IgA toward merozoite antigen is a potent inducer of ROS (65), but IgA's importance in ROS production in malaria is unclear as IgG depletion from serum appears to be sufficient to eliminate most ADRB toward merozoite antigen (52). Further research is needed to identify the characteristics of antibody responses to malaria antigens that effectively elicit neutrophil ADRB.

Evidence for NETS in Malaria

Neutrophil extracellular trap (NET) formation has evolved as an important innate strategy for killing extracellular pathogens, and occurs when activated neutrophils degranulate and release neutrophil antimicrobial factors into the extracellular environment. NETs are mesh-like extracellular structures made up of decondensed chromatin and histones decorated with different antimicrobial granular proteins that can capture, neutralize and kill a diversity of microbes (66). There are several factors which might induce NET formation during *Plasmodium* infections (**Figure 1C**). Crystal uric acid [a potent inducer of NETosis (67)] and its precursor hypoxanthine are released upon iRBC rupture [reviewed in (68)]. In addition, cytokines such as TNF and IL8 which are increased during *Plasmodium* infections [reviewed in (69)], and H_2O_2 secreted by immune cells stimulated by *Plasmodium spp.* antigen [reviewed in (70)] have been shown to induce NETosis *in vitro* [reviewed in (67)] (**Figure 1C**). NET-like structures which stain positive for DNA with 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) have been found in peripheral blood of children with uncomplicated *P. falciparum* infections (71), however it is unclear if these structures were produced in response to *Plasmodium* antigen (as opposed to another stimulus) and/or if they are derived from neutrophils (as opposed to monocytes) (72). There is some qualitative evidence that neutrophils can produce NETs in response to *P. falciparum* antigen *in vitro* (73) however, quantitative evidence that neutrophils produce

NETs in response to *P. falciparum* and evidence that NETs are present in tissues and contribute to pathology of *Plasmodium* infection in humans is still lacking. When brain tissue sections from 4 children with fatal cerebral malaria (CM) and associated retinopathy and 5 with CM without retinopathy (indicating an alternative diagnosis) were stained with antibodies toward NET markers neutrophil elastase and citrillinated histones, no NETs were seen (14). There are no published studies looking for NETS in other human tissues. Possible explanations for the lack of evidence of NETs in *ex-vivo* brain tissue include neutrophils making NETs that the parasites break down. *P. falciparum* asexual blood stage parasites expresses a DNase virulence factor which may be able to break down NETs, as they are made of DNA (74) (**Figure 1C**). Alternatively, it may be that NETs are simply not being formed. If neutrophils phagocytose bulky antigens via opsonisation and/or phosphatidylserine (PS) exposure sometimes they cannot make NETs [reviewed in (75)] (**Figure 1C**) and we know during malaria infection neutrophils do phagocytose bulky antigens (such as merozoites and iRBC) by these mechanisms (**Figure 2**) (37, 39, 76). Further, research is needed to clarify whether NETs do play an active role in *Plasmodium spp*. infections.

Neutrophils, Severe Malaria and Parasite Adhesion

As well as playing a role in parasite clearance, neutrophils may contribute to the pathology of severe malaria syndromes, but there is limited supporting data from human studies on this topic. In CM, neutrophil proteins in plasma [including neutrophil primary granule proteins, neutrophil elastase, myeloperoxidase and proteinase 3 (PRTN3)] are associated with disease (14) and it has been hypothesized that neutrophil products contribute to CM pathology, with elastase damaging the endothelium (77, 78), and inflammatory factors such as TNF [reviewed in (79)] and ROS [reviewed in (80)] increasing expression of the iRBC adhesion receptor, ICAM-1 (81) on endothelial cells to promote parasite adhesion. Also, PRTN3 can cleave endothelial protein C receptor (EPCR) from endothelial cells and contribute to the procoagulant state observed in severe malaria [reviewed in (14)]. On the other hand neutrophil products may also reduce parasite adhesion, for example by PRTN cleavage of EPCR (82) as it is a receptor for parasites associated with severe malaria (83). Also, neutrophil elastase, released by activated neutrophils, can cleave iRBC antigens involved in *P. falciparum* iRBC cytoadherence to C32 melanoma cells (84).

It has also been suggested that neutrophils play a role in the pathology of the liver during *Plasmodium spp.* infections (85). Neutrophil activation by type I Interferon is associated with increased serum levels of transaminases in *P. vivax* malaria and together with murine data showing type I IFN modulates neutrophil migration to the liver in mice, suggests type I IFN are responsible for neutrophil mediated liver pathology in malaria (85). Human evidence showing that neutrophils are present in the liver during infection and could cause damage is lacking. Further studies using human samples to identify the role of neutrophils in severe malaria are needed.

Factors Inhibiting Neutrophil Responses

Although neutrophils can clear parasites in a variety of ways, several mosquito and parasite antigens can modify neutrophil responses to the parasite's advantage (**Figure 2**). For example, mosquito salivary proteins can alter neutrophil function. Secretion of the protein agaphelin in the mosquito's salivary glands is increased upon infection with *P. falciparum*, and agaphelin can inhibit neutrophil elastase activity, neutrophil chemotaxis and NET formation in response to phorbol myristate acetate (PMA) (31) (**Figure 2**). Other salivary proteins that may also alter neutrophil function include the antigen-5 salivary proteins, which scavenge superoxide and inhibit neutrophil ROS (86) (**Figure 2A**).

Parasite antigens which can inhibit neutrophil function include histamine-releasing factor (an ortholog of mammalian histamine-releasing factor), which was shown to inhibit neutrophil IL6 production in the liver in a murine malaria model, and subsequently promoted liver stage parasite development (87). Also, *P. falciparum* protein MSP1-19 can block neutrophil responses to proinflammatory protein S100P, inhibiting neutrophil chemotaxis *in vitro* (32) (**Figure 2B**). There is clearly an interesting dynamic between neutrophils and *Plasmodium*, with each trying to take advantage and control the other. Identification and understanding of neutrophil parasite interactions may result in the identification of novel therapeutic targets and warrants further investigation.

Parasite-neutrophil interactions may also result in susceptibility to other diseases. Malaria infection (especially with severe anemia) has been associated with susceptibility to non-typhoidal salmonella (NTS) bacteraemia (88, 89) and this is thought to occur due to impaired neutrophil function. *In vitro* observations suggest that neutrophil phagocytosis of parasite products results in them being less capable of phagocytosing bacteria (47). *Ex vivo* observations show reduced ability for neutrophils to generate ROS during malaria (18), and this reduced function was associated with increased haemolysis and heme oxygenase-1 (HO-1) expression in infected individuals (18) (**Figure 2A**); similar findings have been reported in asymptomatic infection (90). Mouse data suggests that haemolysis during infection induces HO-1 and results in impaired maturation of neutrophils (91). In addition mouse data shows that IL10 [which is also associated with infection in children (90)] inhibits neutrophil migration resulting in altered clinical presentation of NTS (92).

Pigmented Neutrophils as a Marker of Disease Severity

Neutrophils with malaria pigment can be seen in the peripheral blood during *Plasmodium* infection. In children, the percentage of neutrophils with pigment in the peripheral blood increases with disease severity (93, 94) and is positively correlated with parasitaemia (93, 95). The percentage of neutrophils with pigment has also been positively associated with mortality due to severe malaria in adults and children (94, 96), and in parasitaemic pregnant women high numbers of pigmented neutrophils in the peripheral blood during gestation were associated with

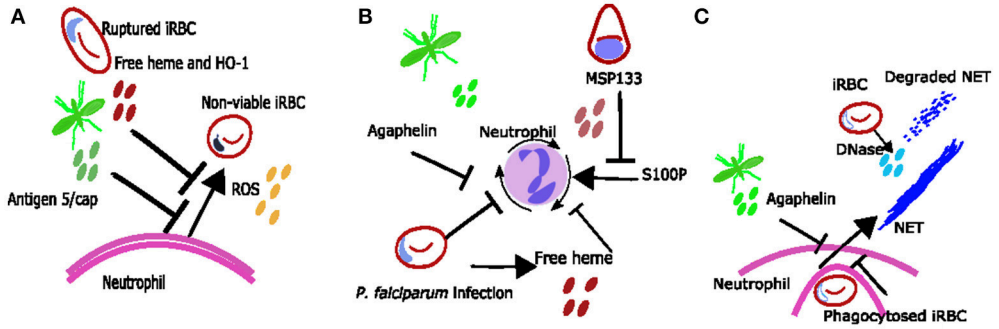

FIGURE 2 | Proposed mechanisms neutrophil inhibition during *Plasmodium spp.* infection. **(A)** Neutrophil ROS may be inhibited due to excess free heme and HO-1 or due to mosquito salivary protein antigen 5/cap. **(B)** reduced neutrophil chemotaxis may be due to exposure to free heme, merozoite protein MSP133 or mosquito salivary protein agaphelin. **(C)** It is possible that phagocytosis of bulky parasite antigens inhibits neutrophils from producing NETs during *Plasmodium spp.* infections and that any NETs produced are degraded by parasite DNase.

lower birth weights at delivery (97). The predictive value of pigmented neutrophils (94, 96, 97) suggest that they could indicate sequestered biomass, and because pigment-containing neutrophils are cleared from the circulation after about 72 h (29) it is likely they are a marker of recent pigment phagocytosis. Pigment containing neutrophils have also been associated with disease severity in *P. knowlesi* malaria (98).

Neutrophils and Antimalarials

A number of *in vitro* studies have investigated whether neutrophil activity may be affected by antimalarial drugs. At non-physiological levels chloroquine, quinine, proguanil, and mefloquine all inhibit neutrophil oxidative burst (99) however at lower physiological plasma levels they, as well as sulphadoxine, cycloguanil, pyrimethamine and tetracycline, have no depressive effects on oxidative burst nor markers of oxidative metabolism (99). Likewise at high concentrations, chloroquine decreases the phagocytic activity of neutrophils and quinine, chloroquine and quinacrine inhibit neutrophil chemotaxis but at physiological concentrations they do not (100, 101). There is however some evidence that neutrophil chemotaxis and neutrophil iodination (a measure associated with phagocytosis and oxidative burst) are inhibited at physiologically relevant concentrations by pyrimethamine and by mefloquine or pyrimethamine respectively, (101). Mefloquine inhibits oxidative burst by interacting with cellular phospholipid-dependent protein kinase C (102). Amodiaquine and pyronaridine have been shown to cause neutrophil glutathione depletion in *in vitro* systems where pyronaridine is oxidized to a quinonimine metabolite, raising concerns they could be cytotoxic to neutrophils at physiological concentrations (103). However, *in vivo* studies in rats did not find any quinoneimine metabolites after receipt of pyronaridine nor is there is clinical evidence of significant toxicity associated with pyronaridine use in humans [reviewed in (104)].

As well as effects on neutrophil function, studies have raised the possibility that neutrophil numbers may be affected by antimalarials (105–107). In an analysis of data from 7 randomized trials across 13 sites in 9 countries comparing artesunate-amodiaquine to single and combination treatments (including amodiaquine mono-therapy, artesunate mono-therapy, artemether-lumefantrine, artesunate and sulphadoxine-pyrimethamine, and dihydroartemisinin) the treatment-emergent adverse event incidence of neutropenia was 11% (107). However, when neutrophil counts were compared between treatment groups there was no apparent differences (107). Interestingly, in the case of artesunate, an effect on neutrophil numbers may be dose dependent. In a clinical study conducted among Cambodian patients with uncomplicated malaria, those who received 6 mg/kg/day had lower neutrophil counts than those who received 2 or 4 mg/kg/day, also 5 of 26 patients who received 6 mg/kg artesunate developed neutropenia $<1000/mm^3$, while only 1 of 38 patients receiving 2 or 4 mg/kg/day did (105).

A Brief Overview of Neutrophils in Animal Models of Malaria

Murine models have been used to study the role of neutrophils in malaria complications including lung injury, CM and liver injury. Accumulation of neutrophils in the lungs has been associated with lung injury in multiple murine malaria models (73, 108–114) and lung injury is associated with increased TNF (108, 109), parasite sequestration in the pulmonary vasculature (113), the presence of NETs (73), neutrophil adhesion to endothelial walls, and with increased vascular permeability (110, 111) but not ICAM-1 expression (114).

In murine models of CM, neutrophils have been shown to express cytokines IL2, IL12p40, IL18, IFNγ, and TNF as well as chemoattractive-chemokines monokine-induced by gamma (MIG), macrophage inflammatory protein-1α (MIP1α) and IFNγ induced- protein 10 (IP10) (115) suggesting a role for neutrophils in cytokine and chemokine secretion. In murine CM neutrophils are detected in the microvasculature (116), and neutrophil depletion results in decreased monocyte sequestration and microhaemorrhages in the brain and prevents development of CM (117). Other neutrophil factors associated with CM include neutrophil secretion of CXCL10 which may contribute to high parasitemia and disease (118), and neutrophil expression

of FcεRI (a high affinity IgE receptor) which may result in the production of proinflammatory cytokines (119).

Studies have identified a possible role for neutrophils in liver damage in murine models with neutrophil dependent liver damage being associated with free heme, NFkB activation and neutrophil infiltration (120). Neutrophil infiltration and subsequent liver damage may also be dependent on type I IFN signaling (85).

Murine models can play a valuable role in dissecting the role of neutrophils in disease, but whether the roles of neutrophils in humans and murine malaria are similar is still unclear and more work needs to be done to validate findings in humans.

A Role for Neutrophils in Vaccine Mediated Immunity

Neutrophil-antibody interactions can play an active role in parasite clearance and should be considered when evaluating vaccine mediated immunity. ADRB toward merozoite antigens is associated with protection (52, 56), but whether other antigens such as those on the asexual iRBC, gametocytes or sporozoites can trigger ADRB, or whether such responses could be associated with protection, is unknown. Likewise, although it is clear that neutrophil phagocytosis of parasite antigen, merozoites, iRBC and possibly gametocytes occurs *in vivo* (35, 36, 40, 121), it is unknown whether antibodies promoting neutrophil phagocytosis are protective. Studies examining antibody mediated neutrophil functions to a variety of antigens and their associations with protection will help elucidate the role neutrophils have in antibody mediated immunity and their potential in vaccine mediated immunity.

A few studies have used animal models to investigate the role of antibody-dependent neutrophil responses in the context of protection from disease and also vaccination. In one study, murine IgG1 toward MSP1-19 was effective at inducing human neutrophil ADRB and degranulation, but these same antibodies did not protect against *P. berghei* expressing MSP1 *in vivo* (122). In another study, serum from previously challenged mice and both murine IgG1 and IgG2 could elicit ADRB from murine neutrophils in response to murine antigens *in vitro*, however vaccination of mice with MSP1-42 resulted in antibodies which did not elicit neutrophil ADRB toward merozoites and ADRB did not contribute to vaccine mediated protection (123). On the other hand, when non-human primates were immunized with MSP-1, antibodies were produced which opsonised merozoites and elicited ADRB by neutrophils (63), suggesting that vaccination can result in generation of antibodies which activate neutrophils.

As well as their role in parasite clearance by antibody dependent mechanisms, neutrophils may also play a role in immune responses to vaccines. There is growing evidence that neutrophils have the capacity to present antigen to T-cells. Whilst they may not be as effective at antigen presentation as typical antigen presenting cells their overall impact may be significant due to their sheer numbers [reviewed in (4)]. At least in the case of irradiated sporozoites, data from murine models suggests that neutrophils are available to take up the vaccine as intradermal injection of both irradiated and wild type sporozoites result in recruitment of neutrophils (and inflammatory monocytes) to the injection site, however there was no evidence that neutrophil depletion in this model affected the establishment of a protective immune response (124). The occurrence and significance of antigen presentation by neutrophils during both natural malaria infections and vaccination is yet to be investigated.

FUTURE DIRECTIONS

Neutrophils in malaria remains understudied, undertaking the future research priorities listed below will go a long way in helping us to understand the role of neutrophils during *Plasmodium spp.* infections.

1. Identify the presence (or absence) of neutrophils at sites of parasite sequestration using *ex vivo* samples from humans.
2. Identify neutrophil subsets during infection in humans using *ex vivo* samples from humans.
3. Identify and quantify neutrophils products (including NETs) at sites of parasite sequestration and in the periphery using *ex vivo* samples from humans.
4. Investigate possible roles of neutrophils in asymptomatic *Plasmodium spp.* infection, clinical malaria and severe malaria by comparing neutrophil counts and indicators of neutrophil activation and/or inhibition between different clinical groups.
5. Using *in vitro* models investigate the role of parasite products (such as DNAse) on neutrophil function.
6. Measure antibody mediated functions of neutrophils (such as ADRB & phagocytosis) and investigate their associations with protection from disease.
7. Using *in vitro* models clarify the role of complement in neutrophil parasite interactions.
8. Identify whether neutrophils should they be considered in evaluation of antibody mediated immunity provided by vaccines.
9. Investigate the role of neutrophils in antigen presentation in the context of both natural infection and vaccination.

CONCLUSION

The role of neutrophils in protection and disease during *Plasmodium spp.* infections has been little studied, and important questions remain. Further research with a focus on neutrophil responses toward the parasite and how neutrophils play a role in parasite clearance will likely aid in the development and evaluation of vaccines for malaria.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Human TLR8 Senses RNA From *Plasmodium falciparum*-Infected Red Blood Cells Which Is Uniquely Required for the IFN-γ Response in NK Cells

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During blood-stage malaria, the innate immune system initiates the production of pro-inflammatory cytokines, including IFN-γ, that are critical to host defense and responsible for severe disease. Nonetheless, the innate immune pathways activated during this process in human malaria remain poorly understood. Here, we identify TLR8 as an essential sensor of *Plasmodium falciparum*-infected red blood cells (iRBC). In human immune cells, iRBC and RNA purified from iRBC were detected by TLR8 but not TLR7 leading to IFN-γ induction in NK cells. While TLR7 and 9 have been shown to lead to IFN-γ in mice, our data demonstrate that TLR8 was the only TLR capable of inducing IFN-γ release in human immune cells. This unique capacity was mediated by the release of IL-12p70 and bioactive IL-18 from monocytes, the latter via a hitherto undescribed pathway. Altogether, our data are the first reported activation of TLR8 by protozoan RNA and demonstrate both the critical role of TLR8 in human blood-stage malaria and its unique functionality in the human immune system. Moreover, our study offers important evidence that mouse models alone may not be sufficient to describe the human innate immune response to malaria.

Keywords: innate immune system, toll-like receptor 8, interleukin 12p70, interleukin 18, interferon gamma, NK cells, malaria, *Plasmodium falciparum*

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INTRODUCTION

With 216 million clinical cases and over 400,000 deaths worldwide in 2016, malaria remains a significant global health problem (1). Of these fatalities, over two thirds occur in children under five, and 99% are due to infection with *Plasmodium falciparum* (*P. falciparum*). Severe falciparum malaria is associated with a strong pro-inflammatory response during blood-stage disease (2–5). This release of pro-inflammatory cytokines, including IL-1β, IL-12, IL-18, TNF, and IFN-γ, is seen as a "double-edged sword," correlating with both protective immunity and disease severity (6–8). Despite important differences between murine and human malaria, murine models of malaria

using rodent *Plasmodium* species recapitulate the pro-inflammatory phenotype observed in human infection (9–11) and have demonstrated a critical dependence on early IFN-γ release from natural killer (NK) cells for host defense during the blood stage (12–15). Further studies have reported that IFN-γ release during murine malaria occurs downstream of TLR7 and TLR9 signaling (16, 17) and is thus dependent on the sensing of *Plasmodium*-derived nucleic acids. Human NK cells have been observed to produce IFN-γ upon exposure to *P. falciparum*-infected red blood cells (iRBC) (18, 19), a process which is dependent on IL-12p70 and IL-18 release from accessory cells (20). In addition to NK cells, a number of reports have demonstrated that CD4+ and CD8+ αβT cells and, in particular, γδ T cells produce IFN-γ after Plasmodium infection (21–23). However, despite a broad variety of well-characterized *Plasmodium*-derived pathogen-associated molecular patterns (PAMPs) which activate known pattern recognition receptors (PRRs) (6, 24), it remains unclear which precise PAMPs and PRRs are required for IFN-γ release from human cells in the blood stage of *Plasmodium* infection.

Of note, there are important differences between murine and human innate immune sensing of nucleic acids in the endosome. While murine TLR7 and TLR9 are expressed in a broad variety of myeloid cells and their activation lead to the release of type I interferons (IFNs) and MyD88-dependent NF-κB driven cytokines, including the NF-kB/IRF1 cytokine IL-12p70, human TLR7 and TLR9 expression is largely restricted to B-cells and plasmacytoid dendritic cells (pDCs), and their activation lead to the release of type I IFNs (25–27). IL-12p70 release from human monocytes can instead be triggered by the endosomal RNA-sensor TLR8 (28, 29), which, in mice, does not function as a pro-inflammatory single-stranded RNA receptor (30, 31) and may, in fact, have an anti-inflammatory function (32, 33). Human TLR8 shares many common RNA and small-molecule ligands with TLR7, yet differential activators of TLR7 and TLR8 have been described (29, 34, 35), and recent studies utilizing CRISRPR/Cas9 genome editing in human cells have shown that human TLR8 can preferentially recognize bacterial RNA and initiate antibacterial host defense (36, 37). However, due to the lack of murine models for TLR8 function to date, we are only beginning to understand the functionality of TLR8 in the human system (38, 39).

In this study, we demonstrate that, in contrast to murine models, *P. falciparum*-derived RNA (PfRNA) but not DNA (PfDNA) is able to induce IFN-γ release from human NK cells. Moreover, our data reveal that PfRNA and *P. falciparum*-infected red blood cells (iRBC) and, in particular, PfRNA are selectively sensed by human TLR8, not TLR7, in the endolysosomal compartment, the first description of the participation of TLR8 in the sensing of eukaryotic pathogens. We provide evidence that TLR8 activation in human monocytes is essential for IFN-γ release from natural killer (NK) cells upon stimulation with PfRNA and iRBC, thus elucidating the PAMPs triggering early IFN-γ release in human malaria. Human TLR other than TLR8 were unable to induce IFN-γ in NK cells, since only iRBC, PfRNA, and other TLR8 ligands were able to both induce IL-12p70 and simultaneously prime and activate the release of bioactive IL-1β and IL-18. Thus, our study underscores the unique role of TLR8 in human host defense.

RESULTS

P. falciparum-Infected Red Blood Cells and *P. falciparum* RNA but Not DNA Induce IFN-γ Release From Human NK Cells

TLR7 and TLR9 have been reported to contribute to innate immune sensing during blood-stage infection in murine malaria models. Whereas, *P. falciparum*-derived DNA is a known human TLR9 ligand (40, 41), the pathways involved in the sensing of *P. falciparum* RNA (PfRNA) and *P. falciparum*-infected red blood cells (iRBC) via human endosomal PRRs has not been investigated to date. To analyze this, we incubated human PBMC with or without depleted NK cells with iRBC (purity of depletion **Supplementary Figure 1**). As previously published (18, 19), iRBC-induced IFN-γ production in human PBMC and was substantially dependent on NK cells (**Figure 1A**). To determine the role of plasmodium DNA (PfDNA) and RNA (PfRNA) in this IFN-γ response and which PRRs are involved, we purified PfDNA and PfRNA from iRBC. A striking difference could be observed after direct stimulation with these iRBC-derived nucleic acids: only PfRNA but not PfDNA was able to induce IFN-γ in PBMC, thus implicating RNA but not DNA-sensing PRRs to be involved in IFN-γ release from PBMC in response to *P. falciparum* (**Figure 1B** and **Supplementary Figure 2**). As analyzed by flow cytometry, the cell subsets that are responsible for this IFN-γ release were mainly found to be NK cells and to a lesser extent NKT cells and γδT cells (**Figures 1C,D**). This is in line both with previous reports (18, 22) and the partial reduction of IFN-γ in PBMC in response to iRBC after depletion of NK cells seen in **Figure 1A**. Activation of γδT cells also requires the γδT-cell receptor, and numerous publications demonstrate the importance of the NK cell for the early immune response in the blood stage (12, 14, 18, 19). Thus, in the current manuscript, we chose to focus on the NK-cell response after exposure to plasmodial PAMPs. We additionally compared the expression of other markers of NK cell activation after stimulation with PfDNA and PfRNA. CD69 was robustly upregulated after treatment with PfRNA in NK cells but only weakly induced in response to PfDNA (**Figure 1E**). However, the release of cytotoxic granules was induced by both PfRNA and PfDNA in a comparable fashion (**Figure 1F**). Pathogenic RNA can be sensed by a number of cytosolic and endolysosomal PRRs (26). Thus, to determine whether PfRNA was sensed within the cytosolic or endosomal compartment, we treated human PBMC with chloroquine or bafilomycin before stimulating with iRBC or PfRNA. Chloroquine (CQ) and bafilomycin inhibit lysosomal acidification and thus the activation of TLRs 3, 7, 8, and 9 within the endosome of immune cells (42, 43). Both chloroquine and bafilomycin inhibited the induction of IFN-γ in response to iRBC and PfRNA (**Figure 1G**). A possible contamination with endotoxin could be excluded in a LAL assay (**Supplementary Figure 3**). Thus, our data demonstrate that IFN-γ is induced from human NK cells by PfRNA but not

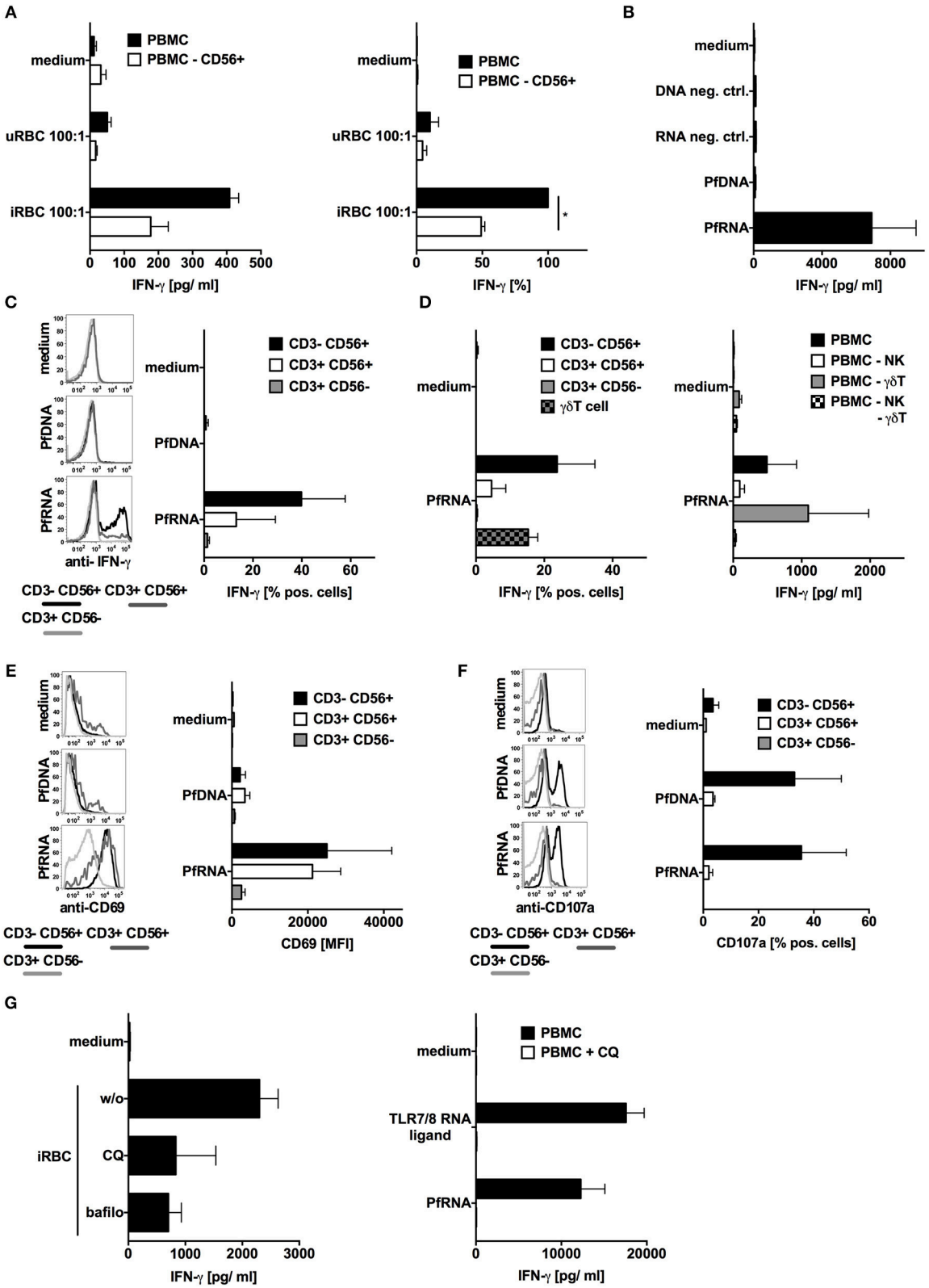

FIGURE 1 | *P. falciparum*-infected red blood cells (iRBC) and *P. falciparum*-RNA (PfRNA) but not DNA (PfDNA) induce IFN-γ in human PBMC via endosomal PRRs. **(A)** Human PBMC with or w/o depletion of CD56+ cells were stimulated with iRBC or uninfected red blood cells (uRBC) as indicated. Twenty-four hours later, IFN-γ

(Continued)

FIGURE 1 | was analyzed in the supernatant. One representative donor depicted left, mean ± SEM of 5 donors in relation to iRBC on PBMC right. **(B)** Human PBMC were stimulated with PfRNA, PfDNA, or an inert RNA or DNA (neg. ctrl.). Twenty-four hours later, IFN-γ was analyzed in the supernatant. Graph shows mean ± SEM of 5 donors. **(C)** Human PBMC were stimulated with PfRNA or PfDNA, after 12 h cells were harvested, fixed, and IFN-γ expression in CD3-CD56+, CD3+CD56+, and CD3+CD56- cells was analyzed by flow cytometry. Percentage of positive cells is depicted. Graph shows mean ± SEM of $n = 6$ donors/$n = 4$ donors for PfDNA. **(D)** Done as described for **(C)** but additional γδT cells were analyzed (left graph, mean ± SEM $n = 3$) and PBMC with depleted cell subtypes as indicated were incubated for 24 h with PfDNA or PfRNA before IFN-γ was analyzed in the supernatant (right graph, ±SEM $n = 4$). **(E)** Done as described for **(C)** but cells were analyzed for surface expression of CD69 and mean fluorescence intensity is depicted. Graph shows mean ±SEM of 4 donors. **(F)** Done as described in **(C)** but after 12 h cells were blocked with Brefeldin A, incubated with 5×10^4 tumor cells (A549) and analyzed by FACS for CD107a expression. Graph shows mean ± SEM of 2 donors **(G)** Human PBMC were treated with 10 μM chloroquine (CQ) or 50 nM bafilomycin (bafilo) for 1 h and then stimulated with RNA (right) or *P. falciparum*-infected (iRBC) or uninfected (uRBC) red blood cells (left) as indicated. Graph shows mean ± SEM of 4 donors. *Indicates a $p < 0.05$.

PfDNA, and PfRNA and iRBC are recognized by an RNA-sensing PRR within the endosomal compartment.

P. falciparum RNA and *P. falciparum*-Infected Red Blood Cells Specifically Activate TLR8

The single-stranded RNA(ssRNA) sensor TLR7 has been reported to act as an early sensor of plasmodium infection (17), and TLR7-deficient mice are partially protected from cerebral malaria (44). In human cells, ssRNA in the endosome can be sensed by both TLR7 and TLR8. Thus, we attempted to determine the respective involvement of these two PRRs in PfRNA and iRBC sensing. To specifically analyze the interaction of PfRNA with TLR7 and TLR8, we utilized HEK293-XL cell lines (Invivogen) overexpressing human TLR7 or TLR8 (HEK-TLR7, HEK-TLR8). PfRNA, human PBMC total RNA and *E. coli* RNA were respectively co-transfected with a gaussia luciferase (gLuc)-based NF-kB reporter into HEK-TLR7 and HEK-TLR8 (**Figures 2A,B**). The small-molecule agonists of TLR7 and 8 (CL075-TLR7/8; CL264-TLR7) and the inert RNA poly$(CA)_{10}$ (45) were used as controls. As expected, a robust gLuc signal in both HEK-TLR7 and HEK-TLR8 cells was observed for CL075; CL264 induced a stronger signal in HEK-TLR7, and human total RNA and poly$(CA)_{10}$ (neg. ctrl.) showed only limited activity. In contrast, PfRNA induced a much stronger gLuc signal in HEK-TLR8 cells (**Figure 2B**) than in HEK-TLR7 (**Figure 2A**), indicative of a selective ability to activate TLR8. Strikingly, this differential activation of TLR8 was even more pronounced than that of *E. coli* total RNA, which has been already described as a preferential ligand of TLR8 (37).

Next, to examine TLR7 and TLR8 activation under endogenous, endosomal conditions, we also generated TLR7-deficient ($TLR7^{-/-}$), TLR8-deficient ($TLR8^{-/-}$), and double-deficient ($TLR7^{-/-}TLR8^{-/-}$) monocytic THP-1 cell lines using CRISPR/Cas9 genome editing technology (46) (**Supplementary Table 1**). For experiments with purified PfRNA and iRBC, cells were then subjected to stimulation with IFN-γ and PMA in order to upregulate TLR7 expression, and, in this setting, both TLR7 and TLR8 activation lead to TNF release (47). The dual TLR7/8 ligand 9.2s RNA (48) was used as a positive control and induced TNF release in WT, $TLR7^{-/-}$, $TLR8^{-/-}$ but not $TLR7^{-/-}TLR8^{-/-}$ THP-1 cells. In contrast, endosomal delivery of PfRNA only induced TNF in wildtype and TLR7-deficient cells, but not in $TLR8^{-/-}$ or $TLR7^{-/-}TLR8^{-/-}$ cell lines (**Figure 2C**). Thus, in line with the differential activation of TLR8 by PfRNA seen in the HEK reporter cell lines, TNF release from THP-1 cells after PfRNA stimulation critically depended on TLR8.

Since PMA-differentiated THP-1 are highly phagocytic (49), we also used the TLR-reporter cell lines to investigate the innate immune response and sensing of PfRNA upon uptake of intact iRBC. We incubated primed THP-1 cells with iRBC, and, as seen with PfRNA, TNF levels were significantly reduced in $TLR8^{-/-}$ and $TLR7^{-/-}TLR8^{-/-}$ but not $TLR7^{-/-}$ deficient cells (**Figure 2D**), thus demonstrating that iRBC are capable of TLR8 but not TLR7 activation. Of note, TNF release was not completely abrogated in TLR8-deficient lines, which may be due to TNF release downstream of TLR2 or TLR4 signaling, as has been described for glycosylphosphatidylinositol (GPI) anchors in *P. falciparum* (6).

Next, we investigated whether we could recapitulate these findings in PBMC and primary human monocytes. To this end, human PBMC were stimulated via endosomal delivery of PfRNA with 9.2s RNA as a TLR7/8 positive control. TLR8 stimulation is known to induce high levels of TNF and IL-12p70 release, while TLR7 activation in human PBMC induces high levels of IFN-α (27). As expected, endosomal delivered 9.2s RNA induced robust activation of TNF (**Supplementary Figure 2A**) and IL-12p70 (**Figure 2E**), indicative of TLR8 activation, and IFN- α, indicative of TLR7 signaling (**Figure 2F**). In contrast, while PfRNA stimulation induced TNF (**Supplementary Figure 4A**) and IL-12p70 (**Figure 1E**), it only resulted in limited induction of IFN-α (**Figure 2F**). Moreover, IFN-α release, but not IL-12p70, was reduced after pDC depletion from PBMC (**Figures 2E,F** and **Supplementary Figure 1**), which is in line with previous studies reporting robust expression of TLR7 on pDC (50, 51). Since human monocytes express high levels of TLR8, CD14+ cells were purified from PBMC and stimulated with 9.2s RNA and PfRNA. As expected, primary monocytes demonstrated robust IL-12p70 induction upon stimulation with both RNA ligands (**Supplementary Figure 4B**).

We then examined whether stimulation of PBMC with intact iRBC resulted in a pattern similar to what was observed for purified PfRNA. We incubated PBMC with iRBC, which led to IL-12p70 induction, in line with previously published data (20) (**Figure 2G**). In addition, we could also confirm previously published data in which depletion of monocytes, but not of pDCs, reduced levels of IL-12p70 release upon exposure to iRBC (**Figure 2G** and **Supplementary Figure 1**). In contrast to endosomal delivery of defined nucleic acids, whole pathogen exposure can potentially activate a variety of PRRs. In particular,

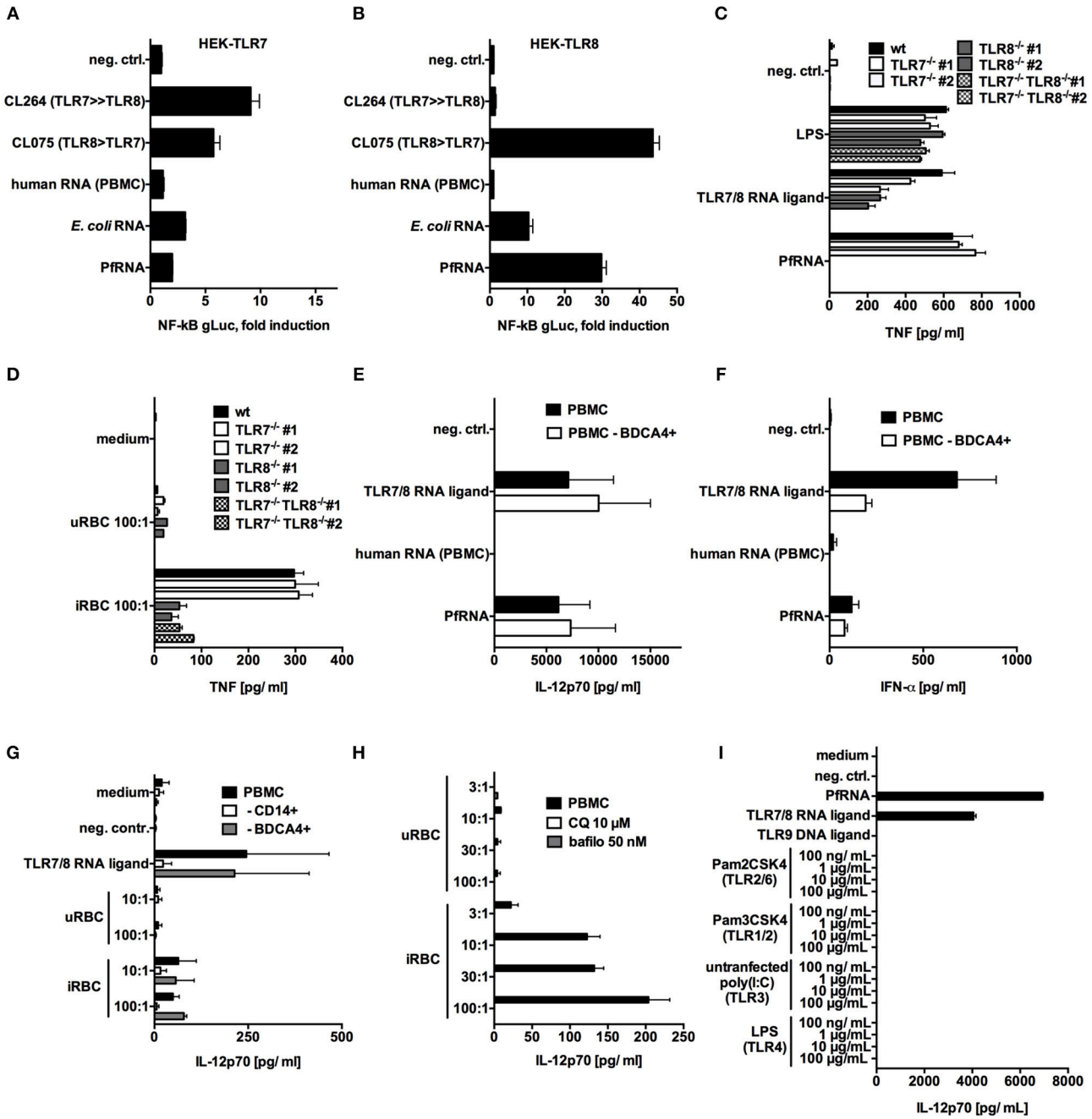

FIGURE 2 | *P. falciparum* RNA (PfRNA) and *P. falciparum*-infected red blood cells specifically activate TLR8. **(A,B)** HEK 293-XL cells overexpressing TLR7 **(A)** and TLR8 **(B)** were transfected with an NF-κB-gLuc reporter plasmid and stimulated with 1 μg/mL of the small-molecule ligands CL264 and CL075 or with RNA as indicated. gLuc activity was measured in cellular supernatants after 16 h. **(C,D)** PMA-differentiated wildtype (wt) THP-1, TLR7$^{-/-}$, TLR8 $^{-/-}$, or TLR7$^{-/-}$ TLR8 $^{-/-}$ cells were primed with 200 U/mL IFN-γ and stimulated with RNA **(C)** or *P. falciparum*-infected (iRBC) or uninfected (uRBC) red blood cells **(D)** as indicated. Cellular supernatants were analyzed for TNF release after 20 h via ELISA. Two clonal cell lines (#1 and #2) were used for each genotype. **(E,F)** Human PBMC and PBMC depleted of cells carrying the pDC marker BDCA4 were stimulated as indicated, and cellular supernatants were analyzed after 24 h for IL-12p70 **(E)** or IFN-α **(F)** via ELISA. **(G)** Human PBMC and PBMC depleted of cells bearing the monocyte marker CD14 or the pDC marker BDCA4 were stimulated as indicated, and cellular supernatants were analyzed after 24 h for IL-12p70 via ELISA. **(H)** Human PBMC were treated with 10 μM chloroquine (CQ) or 50 nM bafilomycin (bafilo) for 1 h and then stimulated with the indicated dilutions (PBMC:RBC) of uRBC or iRBC. Cellular supernatants were analyzed for IL-12p70 after 24 h. **(I)** Human PBMC were stimulated with titrated TLR ligands indicated, and after 24 h, cellular supernatants were analyzed for IL-12p70 via ELISA. **(A–I)** Where indicated, 9.2s RNA served as a TLR7/8 ligand and poly$(CA)_{10}$ as a negative control and CpG 2006 as TLR9 ligand. Unless otherwise indicated, iRBC and uRBC were added in a 100:1 ratio to the cells stimulated, and RNA were complexed with p-L-Arginine before transfection. Data shown are mean ± SD and representative of 3 experiments **(A–D)** or mean ± SEM of 2 **(G)** or 4 **(E,F,H,I)** compiled donors.

Plasmodium RNA from sporozoites is known to activate MDA5 in the cytosol of murine hepatocytes during the liver stage of *Plasmodium* infection (52). Thus, we investigated whether iRBC-induced IL12p70 release was indeed dependent on endosomal sensing. To this end, we used bafilomycin and chloroquine, known inhibitors of endosomal acidification and endosomal TLR signaling (42, 43). Application of bafilomycin and chloroquine abrogated iRBC-induced IL-12p70 (**Figure 2H**), indicating that an endosomal receptor is critically required for IL-12p70 release in response to the sensing of iRBC in human PBMC. Moreover, we investigated the ability of other TLR agonists to induce IL-12p70. Strikingly, only the stimuli with TLR8 agonist activity, 9.2s RNA (TLR7/8) and PfRNA (TLR8), but not other TLRs (TLR1/2, TLR2/6, TLR3, TLR4, TLR9), induced robust IL-12p70 activation (**Figure 2I**), providing a strong indication that other PAMPs in iRBC, such as DNA (TLR9 ligand) or GPI (TLR2/4 agonist), are not able to contribute to iRBC-induced IL-12p70 release via TLR activation in human PBMC. In line with this, DNA derived from iRBC was unable to induce IL-12p70 (**Supplementary Figure 4C**). Altogether, these data indicate that PfRNA specifically activates TLR8 and that iRBC are sensed via *Plasmodium* RNA, leading to TLR8 activation and IL-12p70 release in human PBMC.

Plasmodium RNA and Infected Red Blood Cells Induce the Release of Bioactive IL-1β and IL-18 via TLR8

In addition to IL-12p70, the inflammasome cytokines IL-1β and IL-18 have often been implicated in NK cell activation and IFN-γ release, with bioactive IL-18 originally known as "IFN-γ inducing factor" (53–56). It has also been previously reported that bioactive IL-18 is released from PBMC after exposure to iRBC and participates in IFN-γ induction (20). Thus, we wanted to investigate whether IL-1β and IL-18 release from PBMC after exposure to PfRNA and iRBC were also dependent on TLR8. Moreover, since inflammasome components can also be upregulated or "primed" via TLR stimulation before their posttranslational activation (57, 58), we also specifically examined whether TLR8 only contributed to the initial step of inflammasome priming or also directly to the activation of IL-1β and IL-18, as it has been previously described for TLR4 activation in monocytes (59).

To this end, we isolated human PBMC and incubated them with increasing concentrations of Pam3Cys (TLR1/2 agonist), LPS (TLR4 agonist), 9.2s RNA (TLR7/8 agonist), and PfRNA (**Figures 3A–C**). After priming, cells were stimulated with the potassium ionophore and NLRP3-inflammasome activator nigericin (60, 61). Classical inflammasome priming leads to upregulation of proIL-1β, NLRP3 and other inflammasome proteins but not to IL-1β or IL-18 release (57), which is dependent on their proteolytic activation and non-canonical secretion. While all TLR agonists could prime IL-1β and IL-18 for release after nigericin stimulation, only the TLR4 agonist LPS and the TLR8 agonists 9.2s RNA and PfRNA could directly induce the release of IL-1β (**Figure 3A**) and IL-18 (**Figure 3B**) on their own without addition of nigericin, suggesting that activation of TLR8, like TLR4, could not only prime but also activate the inflammasome. Strikingly, as observed with the TLR4-dependent "alternative inflammasome," TLR8 activation alone did not induce pyroptotic cell death, as demonstrated by a lack of lactate dehydrogenase (LDH) release from stimulated cells (62) (**Figure 3C**). To rule out secondary effects, we isolated primary monocytes and stimulated them with PfRNA, 9.2s RNA, LPS, and iRBC without prior LPS priming, recapitulating what we had observed in complete PBMC and demonstrating that iRBC are capable of direct inflammasome activation (**Figures 3D,E**).

To determine whether this process was indeed dependent on TLR8 itself, PMA-differentiated wildtype and TLR8$^{-/-}$ THP-1 cells were stimulated with 9.2s RNA, PfRNA and iRBC. The inert RNA poly(CA)$_{10}$ and uninfected red blood cells (uRBC) were used as negative control and nigericin as a TLR8-independent NLRP3 stimulus. Once again, 9.2s RNA and PfRNA could directly induce release of IL-1β (**Figure 3F**) and IL-18 (**Figure 3G**), which, strikingly was abrogated in the absence of TLR8. Of note, although it has been reported that the TLR4-dependent alternative inflammasome is not active in THP-1 cells (59), our data demonstrates TLR8-mediated IL-1β and IL-18 release is functional in this cell line.

The alternative TLR4 inflammasome pathway ultimately leads to NLRP3 and caspase-1 activation (59). To investigate a possible role for NLRP3 or caspase-1 in TLR8-mediated inflammasome activation, we used the specific NLRP3 inhibitor (CP-456,773) (63) and the caspase-1 inhibitor YVAD. In THP-1 cells, AIM2 activation via dsDNA served as a NLRP3 independent control (64, 65) and NLRP3 activation via nigericin as a positive control for CP-456,773 activity. Here, like AIM2, TLR8-dependent IL-1β release demonstrated a partial dependence on caspase-1 (**Figure 3H**), but unlike AIM2, a partial dependence on NLRP3 as well. Surprisingly, NLRP3 and caspase-1 inhibition was even less effective in PBMC (data not shown) and purified monocytes (**Figure 3I**), strongly suggesting that TLR8 activates a hitherto uncharacterized, non-canonical, NLRP3-independent pathway leading to both IL-1β and IL-18 release.

TLR8 Activation Is Necessary and Sufficient for IL-12p70 and IL-18 Release and IFN-γ Induction in Response to *P. falciparum*-Infected Red Blood Cells

Our data demonstrate that PfRNA and iRBC induce IFN-γ release from human NK cells downstream from an endosomal PRR (**Figure 1**) and that TLR8 induces IL-12p70, IL-1β, and IL-18 in human monocytes (**Figures 2**, **3**). As it has been previously reported that iRBC can induce IFN-γ from NK cells in a process requiring IL-12p70 and IL-18 (18, 19), we investigated whether TLR8 is the receptor responsible for the release of IFN-γ from human NK cells.

For this, we then analyzed the role of IL-12p70, IL-1β, and IL-18 by blocking their respective signaling pathways using an anti-IL-12 antibody, IL-1 receptor antagonist (IL-1RA) and IL-18 binding protein (IL-18BP) (**Figures 4A,B**). In concurrence

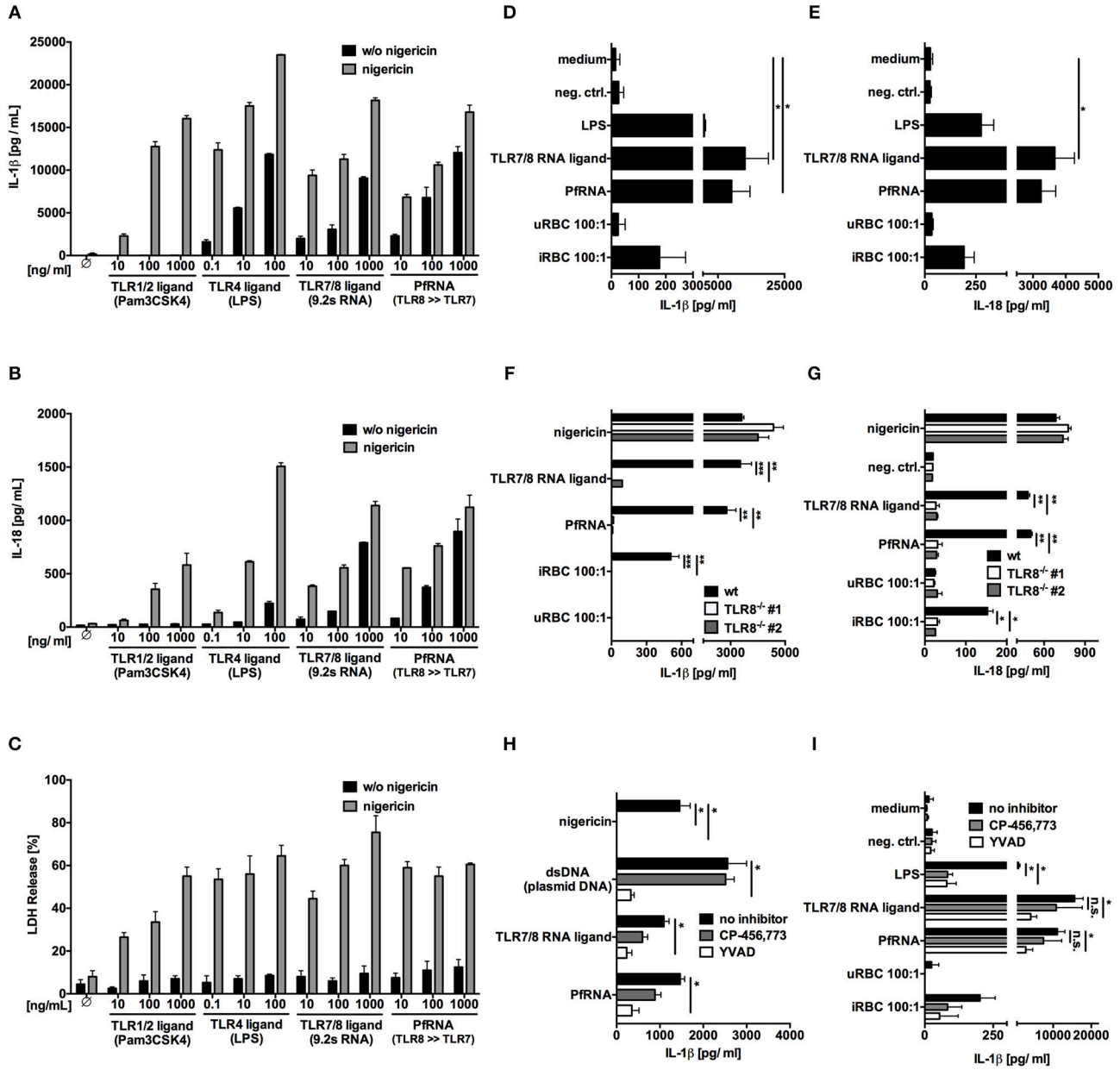

FIGURE 3 | *Plasmodium* RNA (PfRNA) and infected red blood (iRBC) induce the release of bioactive IL-1β and IL-18 via TLR8. **(A–C)** Human PBMC were stimulated with TLR ligands and PfRNA as indicated. After 4 h (LPS, Pam3Cys) or 8 h (RNA ligands), cells were stimulated with 5 μM nigericin, an NLRP3 activator. After 12 h, cellular supernatants were analyzed for IL-1β **(A)** and IL-18 **(B)** release via ELISA and lactate dehydrogenase (LDH) activity **(C)**. **(D,E)** Purified human monocytes were stimulated as indicated. After 16 h, cellular supernatants were analyzed for IL-1β **(D)** and IL-18 **(E)** release via ELISA. **(F,G)** PMA-differentiated wildtype (wt) and TLR8$^{-/-}$ THP-1 were stimulated as indicated. After 16 h, cellular supernatants were analyzed for IL-1β **(F)** and IL-18 **(G)** release via ELISA. Two clonal cell lines (#1 and #2) were used for each genotype. **(H,I)** PMA-differentiated THP-1**(H)** and purified human monocytes **(I)** were incubated with the NLRP3 inhibitor CP-456,773 (5μM) or the Caspase-1 inhibitor YVAD (20μM) for 1 h prior to stimulation. After 16 h, cellular supernatants were analyzed for IL-1β via ELISA. **(A–I)** Where indicated, 9.2s RNA served as a TLR7/8 ligand and poly(CA)$_{10}$ as a negative control. Unless otherwise indicated, iRBC and uRBC were added in a 100:1 ratio to the cells stimulated, and RNA were complexed with p-L-Arginine before transfection. Data shown are mean ± SEM of 4 **(A–E,I)** compiled donors or mean ± SD and representative of 3 independent experiments **(F–H)**. *Indicates a $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

with previously published studies (20, 53), IL-12p70 and IL-18 were both critical factors for IFN-γ induction by PfRNA as well as by iRBC. Moreover, the depletion of monocytes, which produce IL-12p70 and IL-18 upon TLR8 activation, significantly reduced IFN-γ induction whereas depletion of pDCs had no effect (**Figures 4C,D** and **Supplementary Figure 1**).

To elucidate a possible role for other iRBC-derived PAMPs in IFN-γ induction, we tested a broad variety of specific TLR

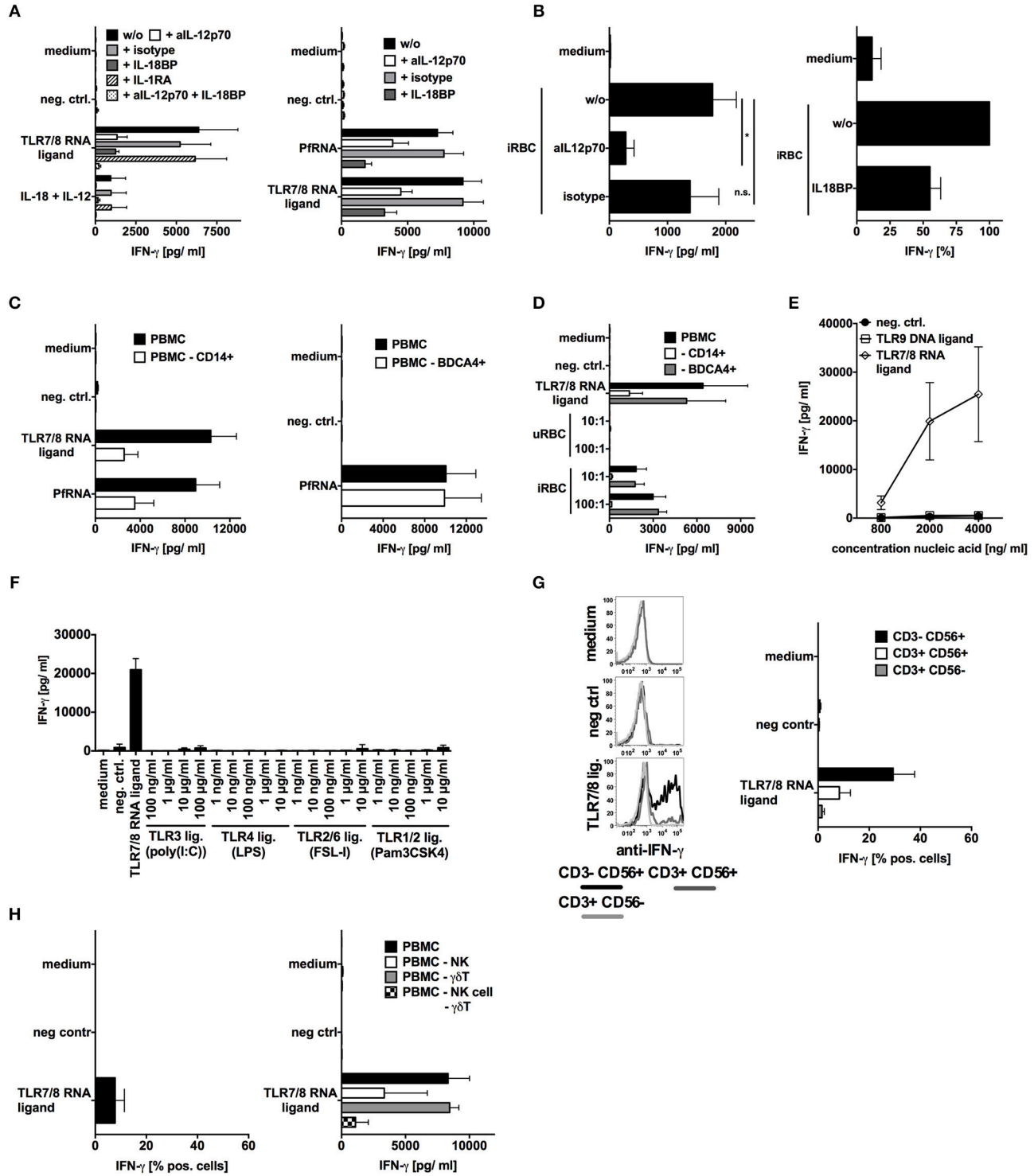

FIGURE 4 | TLR8 activation is necessary and sufficient for IL-12p70 and IL-18 release and IFN-γ induction in response to falciparum should be replaced with *p. falciparum*-infected red blood cells (iRBC). **(A,B)** Human PBMC were treated with inhibitors of IL-12p70 (500ng/mL), IL-1β(10μg/mL) or IL-18BP (100ng/mL) signaling as indicated and then stimulated with TLR ligands and PfRNA as indicated. Addition of recombinant IL-12p70 and recombinant IL-18 served as positive control. **(C,D)** Human PBMC with or w/o depletion of either CD14+ or BDCA4+ cells were stimulated as indicated. **(E,F)** Human PBMC were stimulated with different amounts of TLR ligands as indicated. **(A–E)** Unless otherwise indicated, iRBC and uRBC were added in a 100:1 ratio to the cells stimulated, and RNA ligands were complexed with p-L-Arginine. Where indicated, 9.2s RNA served as a TLR7/8 ligand and poly(CA)$_{10}$ as a negative control and CpG 2006 as TLR9 ligand. All cellular supernatants

(Continued)

FIGURE 4 | were harvested after 24 h and analyzed for IFN-γ release. The data shown are mean ± SEM of 4 compiled donors. **(G)** Human PBMC were stimulated with the TLR7/8 ligand 9.2s RNA or poly(CA)$_{10}$ as negative control. After 12 h cells were harvested, fixed, and IFN-γ expression in CD3-CD56+, CD3+CD56+, and CD3+CD56- cells was analyzed by flow cytometry. Percentage of positive cells is depicted. Graph shows mean ± SEM of $n = 6$ donors. **(H)** Left graph done as described in **(G)** but γδT cells are shown (mean ± SEM of 3 compiled donors), right graph shows IFN-γ release in by PBMC with or without depleted NK cells or γδT cells or both after stimulation with the TLR7/8 ligand 9.2s RNA. *Indicates a $p < 0.05$.

ligands (as in **Figure 2I**) as well as DNA isolated from iRBC (potential TLR9 ligand) for their ability to induce IFN-γ in PBMC. Only TLR8 agonists could induce IFN-γ (**Figures 4E,F**). This is completely in line with our observations that, whereas robust IL-18 production is achieved by TLR4 and TLR8, only TLR8 can robustly induce IL-12p70. Moreover, it supports our previous observation that solely PfRNA but not PfDNA can induce IFN-γ in human NK cells (as in **Figures 1B,C**). Again, as also shown for PfRNA, synthetic RNA activating TLR7/8 induced the strongest response in NK cells but also some IFN-γ release from γδT cells and NKT cells (**Figures 4G,H**). Although our data indicate that TLR8 is critically required for IFN-γ release by NK cells after stimulation with PfRNA, other receptors and cytokines could still possibly modulate this process. Our data also demonstrate, PfRNA can weakly activate TLR7 and induce type I IFN (**Figure 2F**). To characterize the contribution of type-I IFN to type II (IFN-γ) release, we blocked type I IFN signaling using the physiological inhibitor B18R and then stimulated PBMC with a TLR7/8 agonist and PfRNA (**Supplementary Figures 5A,B**). In concentrations of B18R sufficient to abrogate type I IFN recognition by ELISA, there was a small reduction in IFN-γ by B18R in response to the TLR7/8 ligand and PfRNA. This seems to indicate TLR8 induced IFN-γ from NK cells can be boosted by type-I IFN, particularly during simultaneous TLR7 and TLR8 activation. Of note, a broad variety of TLR agonists could not induce IFN-γ in NK cells but could still upregulate activation markers on the surface of NK cells (CD69) and degranulation (CD107a) of NK cells (**Supplementary Figures 6A–E**), in agreement with other publications. However, while TLR9 agonists are capable of inducing IFN-γ in murine splenocytes (**Supplementary Figure 6F**), they do not induce IFN-γ in human PBMC (**Figure 4E**). Thus, our data highlight an important difference in the pathways to IFN-γ induction in mice and humans as well as the unique role of TLR8 in human pathogen sensing.

DISCUSSION

The innate immune system must discriminate between foreign and host molecules in order to effectively recognize pathogenic threats. Ideally, this immune recognition should lead to a host response tailored to the elimination of the specific pathogen. IFN-γ, also known as "macrophage-activating factor," induces a broad range of bactericidal effects and cytotoxic immunity (66, 67). In the context of malaria, but also during infection with intracellular bacteria, IFN-γ facilitates the phagocytosis and elimination of microbes or infected cells, as well as the opsonisation and internalization of iRBC and extracellular pathogens, limiting the spread of infection (7). Recent studies have suggested that TLR8 is critical for the sensing of bacterial RNA (36, 37) and the host defense against several strains of intracellular bacteria (68–70). Our study broadens this repertoire by demonstrating that TLR8 can also detect protozoan RNA, as demonstrated for the intracellular eukaryotic protozoan *P. falciparum*. These data thus underscore the relevance of TLR8 for host defense against intracellular pathogens via IFN-γ induction.

Interestingly, in cultured human immune cells, TLR8 activation but none of the other tested TLR was able to induce IFN-γ in NK cells. This singular connection between TLR8 activation and IFN-γ is the result of the unique ability of TLR8 to induce two key IFN-γ inducing cytokines in human monocytes, IL-12p70 and IL-18, and the induction of these two cytokines represents two particularities of TLR8 signaling. Our results demonstrate that TLR8 is the only human TLR capable of inducing IL-12p70 (**Figure 2I**). IL-12p70 is a dimeric cytokine, consisting of two subunits: IL-12p40, which is regulated by NF-kB and can be transcriptionally activated by many TLRs, including TLR4 and IL-12p35, which is reportedly regulated by IRF1 (71). Little is known about IRF1 activation downstream of TLR8 signaling. Adding to this ambiguity is the fact that many previous papers report LPS-induced production of bioactive IL-12p70, which we could not reproduce when using ultrapure LPS (**Figure 2I**), despite its ability to induce IL-12p40 (data not shown). These differences may be attributable to improvements in LPS purification protocols. Thus, it is will be necessary to use well-characterized ligands when elucidating TLR-specific signaling pathways such as the one leading to the production and release of IL-12p70. It should also be noted that, in our study, we used freshly prepared, intact iRBC to investigate TLR8 activation. In contrast, erythrocyte lysis is necessary for the release of GPI anchors, which activate TLR1/2, TLR2/6 and TLR4. Thus, the storage and treatment of iRBCs may affect which cytokines are produced by iRBC cultures.

The second distinctive feature of TLR8 contributing to IFN-γ-induction is the ability of TLR8 to both prime and activate IL-18 release. Although alternative inflammasome activation has been reported for TLR4 (59), and several publications have described the concerted activation of TLR8 and NLRP3 with distinct steps for priming and activation (72), our study is the first to describe an alternative pathway of direct inflammasome activation by TLR8. Of note, TLR4-dependent alternative inflammasome activation is absent in mice, and murine TLR8 does not induce pro-inflammatory cytokines upon exposure to ssRNA. Thus, the TLR8-IL-1/IL-18 axis contributes yet another difference to the immune sensing between mice and humans, and specifically to the immune sensing of *Plasmodium* parasites in murine and human hosts, which is highly relevant for

the development of future models of malaria in mice, and for further elucidating the immunopathogenesis of malaria in humans.

Another species-specific difference is the response to TLR9. In contrast to murine TLR9, human TLR9 is unable to induce IFN-γ in NK cells in response to a specific ligand or to PfDNA. However, PfDNA does potently induce other cytokines, including type I IFNs. Moreover, a recent study has demonstrated that DNA from extracellular vesicles released by iRBC can also activate the STING/IRF3 pathway leading to type I IFNs release (73). Thus, while PfDNA does not induce IFN-γ, it may still act as an important PAMP during human malaria. Indeed, type I IFNs have been identified as a pivotal immunoregulator of blood-stage malaria (74, 75). In contrast to IFN-γ, type I IFNs were found to suppress innate and adaptive responses in controlled human malaria studies, inducing the anti-inflammatory cytokine IL-10 and limiting IFN-γ release (76). Thus, it will be important to elucidate the precise contribution of STING, TLR9 and type I IFNs to immune sensing of *Plasmodium* parasites in humans and how these pathways act in concert with TLR8 to promote host defense and determine disease severity.

As with all mechanisms of host defense, excessive or chronic activation of innate immune pathways can cause immunopathology and disease. This is particularly true for malaria tropica resulting from *P. falciparum* infection, where the extent of the immune response significantly contributes to the severity and lethality of the disease. In this study, we have identified and characterized a central mechanism by which the malaria parasite *P. falciparum* activates the innate immune system of the host, providing new approaches to controlling and modulating *P. falciparum*-induced inflammation, with the goal of preserving immune defense yet mitigating damage to the host. Here, future studies on the mechanisms of TLR8 signaling may prove crucial to the development of new therapies for malaria and bacterial infections. Moreover, our results highlight the species dependence of the immune response to *Plasmodium* parasites during malaria and the importance of using human immune models in malaria research.

MATERIALS AND METHODS

DNA-, RNA-Oligonucleotides, and TLR Ligands

DNA-oligos CpG2006 (5′-tcgtcgttttgtcgttttgtcgtt; phosphorothioate-linkages) and negative control C20-DNA (5′-ccccccccccccccccccc) were purchased from Metabion (Martinsried, Germany). The following RNA-oligos were used: 9.2s RNA (TLR7/8 ligand 5′-AGCUUAACCUGUCCUUCAA) (48), negative control A20-RNA (5′-AAAAAAAAAAAAAAAAAAAA) [all Biomers (Ulm, Germany)]; *E. coli* LPS was obtained from Sigma-Aldrich (Schnelldorf, Germany), nigericin, polyI:C, ultrapure LPS, Pam2Cys, Pam3Cys, FSL-I, CL075 and CL264 were from InvivoGen (Toulouse, France).

Preparation, Isolation, and Culture of Cells

PBMC were prepared from buffy coats by density gradient centrifugation using Ficoll separating solution (Biochrom, Cambridge, U.K.), and lysis of RBCs was performed using BD Pharm Lyse (BD Pharmingen). Cell viability exceeded 95% as examined by trypan blue staining. 4×10^5 PBMC were cultured in 200 µl RPMI 1640 medium (Biochrom, Berlin, Germany) 10% (v/v) FCS (Thermo Fisher Scientific, Oberhausen, Germany), 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Schnelldorf, Germany) in 96-well plates. $CD14^+$ monocytes, BDCA-4^+ plasmacytoid dendritic cells and NK cells were depleted from PBMC using CD14 and BDCA4-microbeads and NK cell isolation Kit and monocytes were isolated using CD14 microbeads according to the manufacturer's recommendations (all Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry analysis revealed a purity of > 95% for isolated monocytes and a depletion of >90% of γδ T cells or > 95% of NK+ or CD14+ cells (**Supplementary Figures 1A-C**). As already a low number of pDC are already known to release substantial amounts of cytokines, depletion of pDC was also functionally tested via stimulation with a TLR9 ligand followed by measurement of IFN-α release (**Supplementary Figure 1D**). After pDC depletion IFN-α induction was completely lost. THP-1 cells were propagated in RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin (complete RPMI). For TLR7/8 stimulation, THP-1 cells were differentiated into macrophage-like cells by treatment with 300 ng/ml PMA (Sigma-Aldrich, Schnelldorf, Germany) for 4 h, washed three times with PBS and plated at 8×10^4 cells per well in 96-well plates (200 µl volume per well). Forty-eight hours later, cells were washed twice with prewarmed complete RPMI 1640 medium and treated with 200 U/ml IFN-γ for 6h, followed by two additional medium changes and stimulation [adapted from (47)]. TLR7 and TLR8-expressing HEK293-XL cell lines (InvivoGen, Toulouse, France) were cultured according to the manufacturer's protocol.

Gene-Editing of THP-1 Cells

THP-1 cells were electroporated with plasmids expressing EF1-alpha promoter driven Cas9-NLS-2A-EGFP and U6-guideRNAs targeting TLR7 [GATGTCTGGTATGTGGTTAA(TGG)] and TLR8 [GTGCAGCAATCGTCGACTAC(AGG)], respectively, using the Neon Transfection System (Thermo Fisher Scientific, Darmstadt, Germany) at 1,250 V, 50 ms, 1 pulse. Cells were sorted for EGFP expression and single-cell clones were validated by Sanger sequencing of PCR-amplified genomic loci and functional testing with TLR7/8 specific small molecules (Invivogen, Toulouse, France). TLR7/8 double KOs were generated by consecutive targeting of TLR7 and TLR8 (46).

Culture and Isolation of *Plasmodium* Parasites

The 3D7 strain of *P. falciparum* was cultured in continuous culture in petri dishes at 37°C with a gaseous phase of 90% N2, 5% O_2, and 5% CO_2, according to (77). Parasites were cultured

in fresh human red blood cells from A+ donors in RPMI 1640 medium (Sigma-Aldrich, Schnelldorf, Germany) supplemented with 25 mM HEPES, 20 mM sodium bicarbonate, and 10% heat-inactivated human A+ plasma at 10% (v/v) hematocrit. The parasitemia of the infected red blood cells (iRBC) was determined by light microscopy and estimated by Giemsa-stained smears as previously described. Non-synchronized parasite cultures reaching 4–6% total parasitized erythrocytes were used that contained all parasite stages including schizonts (2.5–4% of all iRBC). The cultures were tested by PCR and found to be free of mycoplasma. Freshly prepared iRBC were used for all stimulation experiments without interim storage or freezing.

Purification of Nucleic Acids

Plasmodium cultures with a parasitemia of >5% were used for nucleic acid isolation. Total RNA from *E. coli* DH10B and human PBMC was isolated with Trizol Reagent (Thermo Fisher Scientific, Oberhausen, Germany) and subjected to digestion with DNAse (Thermo Fisher Scientific, Oberhausen, Germany). *P. falciparum* RNA was isolated from parasitized red blood cells using the RiboPure^{TM} RNA Purification kit, blood (Thermo Fisher Scientific, Oberhausen, Germany). The alternate protocol provided by the manufacturer for the isolation of small RNAs with subsequent DNase I treatment was used. Concentrations and absorbance were measured using a NanoVue^{TM} Plus spectrophotometer (GE Healthcare Life Sciences, Solingen, Germany). The integrity of the RNA was determined via Experion^{TM} (Bio-Rad Laboratories, Munich, Germany) analysis using RNA StdSens Chips, following the manufacturer's protocol. RNA used for experiments showed A_{260}:A_{280} values of >2.0 and an RQI of >7. *Plasmodium* DNA was isolated with Trizol Reagent (Thermo Fisher Scientific, Oberhausen, Germany) and subjected to including RNAse A digestion (Thermo Fisher Scientific, Oberhausen, Germany). Concentration and absorbance were measured using a NanoVueTM Plus spectrophotometer (GE Healthcare Life Sciences, Solingen, Germany). DNA used for experiments showed an A_{260}:A_{280} value of ∼1.8.

Stimulation and Treatment of Cells

Cells were stimulated in duplicates with TLR ligands, oligonucleotides, pathogens and pathogen-derived RNA as indicated. One hundred nanograms per milliliters LPS was used if not stated differently. Uninfected red blood cells (uRBC) or RBC infected with *P. falciparum* (iRBC) were added to cells as indicated. TLR7/8/9 ligands were complexed with polyL-Arginine, 5–15 kDa (p-Arg) as delivery agent. In brief, 360 ng p-Arg (Sigma-Aldrich, Schnelldorf, Germany) were added to 200 ng RNA or DNA for 10 min/ RT in 15 µl PBS and if not indicated differently a final concentration of 1 or 4 ug DNA or RNA/ ml was added to human immune cells and 10 ug DNA or RNA/ ml to THP-1 cells. Plasmid DNA (pBluescript) was used for dsDNA and transfected with Lipofectamine2000 (Thermo Fisher Scientific, Oberhausen, Germany) according to manufacturer's recommendations at a final concentration of 1 µg/ml. TLR7- and TLR8-expressing 293 cells were transfected with an NF-κB-gluc reporter plasmid and an EF1-Promoter-SEAP expression plasmid (as transfection control). Twelve hours after transfection, medium was changed and cells were stimulated with TLR ligands and reporter activity was measured after 16 h. To block endosomal acidification and TLR recognition, 10 µM chloroquine (Sigma-Aldrich, Schnelldorf, Germany) or 50 nM bafilomycin (Invivogen, Toulouse, France) were added 1 h before stimulation. To block NLRP3 and caspase-1 activation, CP-456,773/CRID3 (Sigma-Aldrich, Schnelldorf, Germany) and Z-YVAD-fmk (Enzo Life Sciences), respectively, were added 1 h before stimulation. IL-18 was blocked using IL-18BP (kindly provided by P. Bufler and Merck Serono) and IL-1β by IL-1 receptor antagonist (Anakinra, Swedish Orphan Biovitrum AB, Stockholm, Sweden). IL-12p70 was blocked using anti-IL-12p70 (R&D Systems, Wiesbaden-Nordenstadt, Germany). Recombinant IL-12p70 (ImmunoTools, Friesoythe, Germany) and IL-18 (MoBiTec, Göttingen, Germany) were used as controls.

Cytokine Assays

After supernatant collection at the indicated time points, cytokines were measured in cell culture supernatants by the IFN-α module set (Bender MedSystems; Graz, Austria), human IFN-γ/ IL-1β/ TNF alpha or IL-12p70 ELISA set (all BD Pharmingen, Heidelberg, Germany), respectively. IL-18 was determined via alphaLISA from Perkin Elmer (Waltham, USA). All procedures were performed according to manufacturer's recommendations.

Flow Cytometry Analysis

Intracellular staining was performed after incubation with brefeldin A (1 µg/ml, Sigma-Aldrich, Schnelldorf, Germany) for 4 h. Subsequently, the cells were surface stained and fixed of the cells with PBS/ 4% paraformaldehyde. Then cells were permeabilized with 0.5% saponin (Carl Roth, Karlsruhe, Germany) and intracellularly stained with IFN-γ-Alexa647 (BD Bioscience, Pharmingen, Heidelberg, Germany). To determine the expression of the activation marker CD69, the cells were harvested and stained in PBS containing 2% FCS (Thermo Fisher Scientific, Oberhausen, Germany) with CD69-PE-Cy7, CD3-Horizon V450, CD56-PE (BD Pharmingen, Heidelberg, Germany) for 15 min at 4°C in the dark, washed and analyzed via flow cytometry. Degranulation of NK cells was determined by measuring CD107a-PE, CD3-FITC, CD56-APC (BD Pharmingen, Heidelberg, Germany) and Vγ9-Alexa488 (for γδT cell staining (78) as generous gift from Dietrich Kabelitz and Daniela Wesch). Human PBMCs were co-cultured with tumor cells and CD107a-PE was added for 1 h. Degranulation was blocked with 5 µg/ml Monensin A (Sigma-Aldrich, Schnelldorf, Germany) for an additional 3 h. Flow cytometric analysis was performed using the LSR II from BD Bioscience and data were analyzed with FlowJo® (Tree Star, Switzerland).

Statistics

Statistical analysis was performed using non-parametric tests. Wilcoxon matched-pairs signed rank test was used to compare paired groups. In case of multiple comparisons, Friedman test was used and corrected according to Bonferroni. *indicates a $p < 0.05$; ** < 0.01, and *** < 0.001.

ETHICS STATEMENT

Freshly prepared buffy coats and erythrocytes from human donors were obtained from the Institute for Experimental Hematology and Transfusion Medicine, University Hospital Bonn, Germany with the donors' written informed consent. Approval was given by the ethics committee of the University Hospital Bonn (167/11).

AUTHOR CONTRIBUTIONS

CC, BH, EB, TZ, JD-P, MN, and BP performed the experiments. CC, EB, TZ, MS, BK, AH, and GH conceived experiments. JFS, SS, and BS supported experiments with *P. falciparum* material. CC, EB, and GH wrote the manuscript. All authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00371/full#supplementary-material

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γδ T Cells in Antimalarial Immunity: New Insights Into Their Diverse Functions in Protection and Tolerance

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Uniquely expressing diverse innate-like and adaptive-like functions, γδ T cells exist as specialized subsets, but are also able to adapt in response to environmental cues. These cells have long been known to rapidly proliferate following primary malaria infection in humans and mice, but exciting new work is shedding light into their diverse functions in protection and following repeated malaria infection. In this review, we examine the current knowledge of functional specialization of γδ T cells in malaria, and the mechanisms dictating recognition of malaria parasites and resulting proliferation. We discuss γδ T cell plasticity, including changing interactions with other immune cells during recurrent infection and potential for immunological memory in response to repeated stimulation. Building on recent insights from human and murine experimental studies and vaccine trials, we propose areas for future research, as well as applications for therapeutic development.

Keywords: γδ T cells, Vγ9Vδ2 T cells, *Plasmodium*, cytotoxicity, cytokines, immunological memory

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INTRODUCTION

γδ T cells are unconventional T lymphocytes that are increasingly being appreciated for their unique role in integrating the innate and adaptive arms of the immune system. Comprising approximately 2–5% of peripheral blood T cells in healthy adults, they can uniquely recognize a broad range of antigens without the need for major histocompatibility complex (MHC) and can both establish and regulate the inflammatory response. Many of their individual functions—including production of pro-inflammatory cytokines, cytotoxic killing, antigen presentation, promotion of dendritic cell maturation, B cell help, recruitment of other immune cells, and secretion of growth factors—are shared with other immune cell types. However, a unique combination of antigen specificity, tissue distribution, kinetics and functional properties enable γδ T cells to play an essential role in human immunity (1).

Long known to rapidly increase in number following systemic bacterial or parasitic infection (2–5), γδ T cells may also be important in mediating protection against recrudescence and/or reinfection, particularly in the context of malaria (6–9). Recent investigations of γδ T cell function during this disease caused by parasites in the *Plasmodium* genus are providing new insight into the processes underlying acute responses, as well as protection during chronic or recurrent infection. Despite progress in reducing worldwide incidence of malaria over the last decade, malaria remains a major global health problem, accounting for almost 500,000 deaths annually, predominantly in young children and pregnant women in sub-Saharan Africa (10). Improving our understanding of

the inflammatory and immunoregulatory roles of γδ T cells during malaria infection may provide opportunities to manipulate this response therapeutically, potentially via combined targeting of γδ T cells and B or T cell immunity as is currently being pursued for cancer. This review will integrate recent advances in understanding the diverse functions and plasticity of these fascinating cells in malaria. We discuss results from recent human and murine studies, including vaccine trials, and propose open areas for future research and development of novel antimalarial therapeutics targeting γδ T cells.

THE UNIQUE FUNCTIONAL SPECIALIZATION OF γδ T CELLS

Though γδ T cells can carry out diverse innate- and adaptive-like functions, individual cell subsets have more restricted effector properties depending on expression of T cell receptor (TCR) Vγ and Vδ regions and associated tissue location (1). In humans, the Vγ9Vδ2 subset is the most abundant in adult human peripheral blood; approximately 50–90% of circulating γδ T cells express this combination of chains, previously thought to be due to postnatal expansion. However, Dimova et al. recently demonstrated that Vγ9Vδ2 T cells with pre-programmed effector functions were the predominant γδ T cell subset in fetal blood, suggesting that this subset of γδ T cells may be prepared to respond before birth (11). The other major subset of γδ T cells in humans, Vδ1+ γδ T cells, are enriched in mucosal tissues where they sense host stress and stimulate leukocyte responses (12). In mice, γδ T cells are most common in the skin and mucosal tissue (13) and act as the major initial IL-17 producers in various infectious and autoimmune models. Nearly all murine γδ T cells in the epidermal layer of the skin, also known as dendritic epidermal T cells (DETC), express identical γδ TCRs. In other animals like cattle, sheep, and chickens, γδ T cells express highly diverse TCRs regardless of tissue localization (13). These differences between γδ T cell subsets between species are essential to consider when interpreting conclusions from animal models. Subsets of γδ T cells exhibiting different tissue tropism could have adapted to have differential potential for clonal expansion and therefore diverse roles in immunosurveillance.

Differential γδ T cell subsets recognize different ligands; perhaps the best know interaction occurs between the stress-related phosphoantigens (PAgs) and the Vγ9Vδ2 subset (14). PAgs are intermediates of the eukaryotic mevalonate or the prokaryotic non-mevalonate pathway of isoprenoid synthesis; the former includes eukaryotic PAgs that are overproduced in tumor cells [e.g., isopentenyl pyrophosphate (IPP)] while the latter includes PAgs specifically produced by pathogens, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). Importantly, recognition of these antigens is dependent on cell-cell contact involving the TCR but independent of antigen processing via MHC molecules. The potential for diversity in the γδ TCR repertoire is currently under debate, but there is some evidence from deep sequencing of genomic DNA in a few individuals that though the majority of γδ T cells in peripheral blood carry the same germline TCRγ rearrangement, a substantial percentage (20%) have a more diverse TCRγ repertoire (15). Likely, this sequence diversity represents an evolutionary adaptation to bridge the innate and adaptive immune systems: universal sequences shared across individuals likely perform innate-like functions, while the diverse background repertoire plays a more adaptive role, as has been suggested in a recent study describing TCR repertoires within the Vδ2 compartment (16).

Regarding functional attributes of γδ T cells, γδ T cells can play numerous roles in response to infection, including direct anti-microbial roles, recruitment of innate immune cells (e.g., neutrophils, macrophages) and activation of the adaptive immune compartment (14). For example, γδ T cells rapidly expand in response to mycobacterial phosphoantigen (17), mycobacterium infection (18), and vaccination with the tuberculosis vaccine Bacillus Calmette–Guerin (BCG) (18–20). Vγ9Vδ2 T cells can inhibit mycobacterial growth through soluble granyzme A (17, 21), and correlate with clearance of BCG bacteremia and immunity to fatal tuberculosis in BCG-vaccinated macaques (18). In contrast, human immunodeficiency virus (HIV) is associated with a loss of circulating Vδ2+ cells (22) and expansion of Vδ1+ cells that are able to proliferate and produce IFNγ and IL-17 in response to *Candida albicans* (23). Cytomegalovirus has also been shown to induce $V\delta2^{neg}$ populations of γδ T cells, including Vδ1+ cells, and these $V\delta2^{neg}$ cells have antibody-dependent anti-cytomegalovirus activity through a process that is CD16 dependent (24). Finally, γδ T cells display functional plasticity based on pre-programmed features (i.e., class of innate receptor, nature of inflammatory stimuli, and strength of the TCR signal) or more long-lasting effects induced by TCR signaling and environmental cues (1). For example, Vγ9Vδ2 cells responding to *E. coli* can adapt from a primarily cytokine-producing phenotype to a phenotype promoting phagocytosis (25). Clearly, γδ T cells are capable of adopting a variety of anti-microbial functions, but the precise factors influencing this adaptability in specific infections or at different timepoints during infection require further characterization.

The functional attributes and plasticity of the γδ T cell response to malaria resembles responses to other infections in some aspects, but also reflects the unique life cycle and epidemiology of *Plasmodium*. The epidemiology of malaria in many regions leading to recurrent infection over many years, as well as the diversity of mouse models for malaria, provide opportunities to gain insight into how γδ T cells adapt their function following repeated stimulation.

IMPORTANCE OF γδ T CELLS DURING MALARIA: INSIGHTS FROM NATURAL INFECTION AND EXPERIMENTAL MODELS IN HUMANS AND MICE

Whether in children, malaria-naïve adults, or malaria-experienced adults, it has long been known that γδ T cells (in particular the phosphoantigen-responsive Vγ9Vδ2 subset) expand following infection with the most virulent human malaria

parasite, *Plasmodium falciparum (Pf)* (**Table 1**) (26, 29, 30, 47). Furthermore, higher frequencies and malaria-responsive cytokine production of Vγ9Vδ2 cells correlate with protection against subsequent infection in children living in endemic settings (31, 39). These associations, along with cytotoxic, anti-parasitic functions of Vγ9Vδ2 cells observed *in vitro* (48), suggest an important role for these cells in mediating protective immunity to malaria. Though most studies have focused on the Vγ9Vδ2 subset (**Figure 1**), earlier work suggested that Vδ1+, and not Vδ2+, T cells are expanded in the peripheral blood of individuals from endemic regions (28, 30), similar to what has been described in chronic HIV infection (22). Although this observation may be due to relative loss of circulating Vδ2+ T cells in repeatedly infected individuals rather than expansion of Vδ1+ T cells (34), a recent study demonstrated that non-Vγ9Vδ2, IL-10 producing γδ T cells expand among individuals with uncomplicated malaria (38). Together this suggests that expansion of a non-Vγ9Vδ2 immunoregulatory population of γδ T cells may also contribute to naturally acquired immunity.

In malaria-naïve individuals immunized with the attenuated *Pf* sporozoite (PfSPZ) vaccine, expansion and frequency of γδ T cells (again, particularly the Vδ2 subset) was dose-dependent and a better correlate of protection compared to any other cellular immune responses (42, 43, 45). Numbers of memory Vδ2+ T cells similarly correlated with protection in the first PfSPZ trial in a malaria-endemic region; however, additional studies in the mouse led the authors to conclude that these cells were essential for induction of protective CD8+ T cell responses rather than directly exerting effector functions (46). Additional work is needed to further elucidate the mechanism of Vδ2+ γδ T cell-induced protection, as well as to determine whether Vδ2 frequencies could be used as a biomarker for protection in PfSPZ vaccinations in malaria-endemic regions. Furthermore, in a trial immunizing malaria-naïve individuals with non-irradiated PfSPZ combined with chemoprophylaxis (PfSPZ-cVAC), Vδ2+ γδ T cells, including cells expressing memory markers, also expanded in a dose-dependent manner and increased IFNγ expression (44). Together, these data suggest that γδ T cells may be a correlate for both natural and vaccine-induced protection.

Studies in the mouse model have provided convincing evidence for a role for γδ T cells in directly or indirectly mediating killing of blood-stage and/or liver-stage parasites and preventing parasite recrudescence. However, major differences in γδ clones between mice and humans are an important caveat, as are differences between murine *Plasmodium* strains (**Table 2**). No subset corresponding to the human Vγ9Vδ2 subset exists in mice and most early studies examined all γδ T cells without regard to antigen specificity. In mice infected with the *Plasmodium chabaudi* parasite, γδ T cells expand by 10-fold (57, 58). Mice deficient in γδ T cells experience higher parasitemia during acute *P. chabaudi* infection, as well as substantial parasitemic recrudescence (6–8). Depletion of γδ T cells during chronic *P. chabaudi* infection in B cell-deficient mice also resulted in significantly worsened parasitemia (56). An exciting recent study demonstrated that γδ T cells expand and become activated in later stages of *P. chabaudi* malaria (**Figure 2A**)—much later than CD4+ and CD8+ αβ T cell activation—even when acute stages are cleared early by drug treatment. This clonal expansion of γδ T cell occurred primarily in murine blood, spleen, lung and liver, and effectively prevented late-stage parasite recurrence (9).

In the non-lethal *Plasmodium berghei* XAT model, control of parasitemia seems to be at least partially mediated by CD40 signaling and boosting of dendritic cell activation (53). Following vaccination with lethal *P. berghei* ANKA sporozoites, γδ T cells contribute to pre-erythrocytic immunity by recruiting dendritic cells and CD8+ T cells during vaccination (46), but were not required to prevent infection upon blood-stage challenge. However, different results have been obtained utilizing the nonlethal *Plasmodium yoelii* model. In mice lacking αβ T cells, γδ T cells substantially influenced immunity to *P. yoelii* liver stages, but could not rescue immunity to blood stages (50), suggesting that at least in this parasite strain, γδ T cells act as important effectors and their cytotoxicity may become more effective after interaction with CD4+ T cells. This same group showed that mice lacking γδ T cells had significantly higher *P. yoelii* burden in the liver than similarly challenged immunocompetent mice, suggesting a potential role for γδ T cells in the development of pre-erythrocytic immunity (51). These differences could potentially be explained solely by the different murine parasite strains used, as *P. yoelii* irradiated sporozoite vaccination does not induce sterile immunity while *P. berghei* vaccination does. Alternatively, the discrepant results could be explained by differing sporozoite preparations or the depleting monoclonal antibodies used (59, 60).

Finally, murine models have also suggested a role of γδ T cell in disease pathogenesis. Mice depleted of γδ T cells by monoclonal antibody were protected from cerebral malaria (the most severe form of malaria) due to *Plasmodium berghei*, but mice genetically depleted of γδ T cells did not show this effect (52), implying that effective activation of γδ T cells is extraordinarily time-sensitive. Likely, parasite species/mouse model and IFNγ levels at different infection timepoints strongly influence whether γδ T cells contribute to protection or worsened pathogenesis.

RECOGNITION OF *PLASMODIUM* BY γδ T CELLS

Numerous *in vitro* and *in vivo* studies have aimed to shed light on the mechanisms of γδ T cell activation in response to malaria infection. *In vitro*, human γδ (Vγ9Vδ2) T cells can proliferate in response to *Pf*-infected red blood cell (iRBC) lysates or schizont extract (34, 61–63), iRBC culture supernatants (62, 64, 65) and/or intact iRBCs (33, 48, 63, 66) (**Figure 1**). Presumably, these cells are responding to phosphoantigens present in *Pf* asexual blood stages (67), as earlier studies suggested that *Pf* sexual stages do not stimulate Vγ9Vδ2 T cells (68), though it was recently demonstrated that secreted phosphoantigens from iRBCs from all developmental stages (including gametocytes) are capable of stimulating Vγ9Vδ2 cells (69). Activation of Vγ9Vδ2 T cells in response to HMBPP has recently been shown to require butyrophilin 3A1 (BTN3A1, CD277), a type I glycoprotein in the B7 family (70). Though the precise molecular mechanisms

TABLE 1 | γδ T cell responses to human malaria and associations with clinical outcomes.

Author, year	Country	Cohort	γδ T cell subset	Impact of malaria exposure on γδ T cell activation and function	Associations between γδ T cell features and clinical outcomes
STUDIES OF MALARIA-NAÏVE TRAVELERS					
Roussilhon et al. (26)	France	Adults; acute *Pf*	All γδ	Expand after infection and remain elevated for months; subset respond *in vitro* to *Pf* schizont extract	
Howard et al. (27)	France	Adults; acute *Pf*	Vγ9Vδ2	*In vivo* exposure and *in vitro* stimulation associated with increased surface expression of APC-associated markers, induce naive αβ T-cell responses, cross present soluble prototypical protein to antigen-specific CD8+ T cells	
STUDIES OF INDIVIDUALS IN MALARIA-ENDEMIC REGIONS					
Goodier et al. (28)	Benin	Adults and children	Vγ9+ and Vδ1+	Majority of γδ T cells are Vδ1+; Vγ9+ cells not elevated compared to malaria-naïve controls but do proliferate after *in vitro Pf* stimulation	
Ho et al. (29)	Thailand	Age not reported; acute *Pf*	All γδ	Expand after acute infection and remain elevated for several weeks	
Hviid et al. (30)	Ghana	Children; acute *Pf*	Vδ1+	Increase after treatment and produce pro-inflammatory cytokines	
D'Ombrain et al. (31)	Papua New Guinea	Children	All γδ	Produce IFNγ following *in vitro Pf* stimulation	IFNγ from γδ and αβ T cells associated with immunity to symptomatic infection
Cairo et al. (32)	Cameroon	Neonates	Vδ2+	Placental malaria associated with increased proportions of central memory Vγ2Vδ2 cells in cord blood and altered Vγ2 chain repertoire *ex vivo* or after stimulation	
Stanisic et al. (33)	Papua New Guinea	Children	All γδ	Produce TNF, MIP-1β, and MIP-1α following *in vitro Pf* stimulation	Increased TNF from γδ T cells and monocytes associated with severe malaria
Jagannathan et al. (34)	Uganda	Children	Vδ2+	Repeated infection associated with loss and dysfunction of Vδ2+ cells and increased Vδ2 expression of immunoregulatory genes including Tim3, CD57, CD16	Loss and dysfunction of Vδ2+ cells associated with clinical tolerance to infection
Farrington et al. (35)	Uganda	Children	Vδ2+	Frequencies and function lower and CD16 upregulated among children with high prior malaria exposure; antimalarial chemoprevention associated with enhanced Vδ2+ cytokine production	
Hsu et al. (36)	Malawi	Neonates	Vδ2+	Upregulate PD1 shortly after activation; after engagement of PD1 with PDL1, show dampened TNFα production and degranulation	
Schofield et al. (37)	Papua New Guinea	Children	All γδ	Elevated Tim-3+ γδ T cells across whole cohort; IL-12 and IL-18 contribute to upregulation	Higher proportions of Tim-3+ γδ T cells associated with asymptomatic malaria infection
Taniguchi et al. (38)	Laos	Adults and children; uncomplicated malaria	Non-Vδ2	Expand and produce IL-10 and IFNγ	
Jagannathan et al. (39)	Uganda	Children	Vδ2+	*In vivo* proliferative response attenuated with repeated exposure; repeated infection associated with loss and dysfunction of Vδ2+ cells	Higher pro-inflammatory cytokine production associate with protection from subsequent infection as well as increased odds of symptoms once infected

(Continued)

TABLE 1 | Continued

Author, year	Country	Cohort	γδ T cell subset	Impact of malaria exposure on γδ T cell activation and function	Associations between γδ T cell features and clinical outcomes
VACCINATION STUDIES					
Teirlinck et al. (40); Roestenberg et al. (41)	The Netherlands	Malaria naïve adults; controlled-human malaria infection (CHMI) + chemoprophylaxis	All γδ	Produce IFNγ, even a year after infection	Long-term functional responses associated with protection against re-infection
Seder et al. (42); Ishizuka et al. (43)	USA	Malaria naïve adults; attenuated PfSPZ vaccination	Vδ2+	Expand after vaccination	Higher frequencies correlate with protection after CHMI
Mordmüller et al. (44)	Germany	Malaria naïve adults; non-irradiated PfSPZ vaccination + chemoprophylaxis	Vγ9Vδ2	Expand in dose-dependent manner and produce IFNγ	
Lyke et al. (45)	USA	Malaria naïve adults; attenuated PfSPZ vaccination	Vδ2+	Cell frequency increase after each vaccination and show activated phenotype	
Zaidi et al. (46)	Mali	Malaria-exposed adults; irradiated PfSPZ vaccination	Vδ2+		Vδ2+ T cells significantly elevated among vaccinees who remain uninfected during transmission season

underlying BTN3A1 essentiality remain controversial, there is now substantial evidence for a model in which the cytosolic B30.2 domain senses and binds to intracellular pyrophosphates and additional adaptor proteins. A resulting spatial redistribution or conformational change of the extracellular BTN3A1 domain is then recognized via an unknown mechanism by the Vγ9Vδ2 TCR (71–74). Other signaling pathways for human γδ T cell activation involve the TCR interacting with ligands such as F1-ATPase or endothelial protein C receptor, or additional cell surface receptors such as natural killer group 2 member D (NKG2D) receptors or toll-like receptors (TLR). Some murine γδ T cell subsets also appear to have a similar regulation pathway involving non-BTN3A1 butyrophilin-related molecules (75). It is unknown whether such a mechanism occurs during activation of γδ T cells in malaria. In contrast to previous studies suggesting a requirement for cell-cell contact between γδ T cells and parasites in initiating activation (48, 76), Guenot et al. demonstrated that at least for intact iRBCs, BTN3A is not present on the iRBC surface and cell-cell contact is not necessary for Vγ9Vδ2 activation (it may still be required for merozoites) (77). Rather, soluble molecules with characteristics of phosphoantigens seem to be released at the time of *Pf* egress from red blood cells (RBCs), leading the authors to hypothesize that Vγ9Vδ2 activation occurs via presentation by other γδ T cells primarily in microvessels and in the red pulp of the spleen where later stage iRBCs sequester. It will be important to evaluate evidence for this theory, as well as to assess whether myeloid or other cells can present antigen to γδ T cells.

PROLIFERATION, CYTOKINE, AND CYTOTOXIC RESPONSE OF γδ T CELLS IN RESPONSE TO *PLASMODIUM*

The rapid proliferation of γδ T cells in response to malaria infection likely depends on cytokine signaling and interaction with other immune cells (**Figure 1**). Proliferating γδ T cells can then inhibit parasite growth *in vitro* (61, 78, 79). Intact iRBCs seem to generally be more effective than lysed iRBCs at inducing γδ T cell expansion; however, lysed parasites can be made more effective with the addition of IL-2 (63). Similarly, activated CD4+ T cells may be required in the absence of IL-2 but are unnecessary when exogenous IL-2, IL-4 or IL-15 is added (64, 80). Proliferation of γδ T cells in mouse spleen during chronic *P. chabaudi* infection appeared to depend on cytokines produced by CD4+ T cells (55). Additional molecules identified as required for IL-2-mediated survival and proliferation include the Ig superfamily receptor CD28 [demonstrated both with human samples and in the *Plasmodium berghei* mouse model (81)] and monocyte-derived cytokines, such as IL-10, IL-12, and IL-1β (82), which also could increase cytokine production by γδ T cells.

During malaria infection, γδ T cells are a major source of IFNγ (**Figures 1**, **2**), which is the cytokine most commonly associated with protection (83). Early IFNγ production has been associated with protection from clinical malaria in some cohorts (31, 84) but associated with worsened symptoms in others (33). There is some debate over whether γδ T cells or NK cells are the predominant source of IFNγ; several authors have suggested that

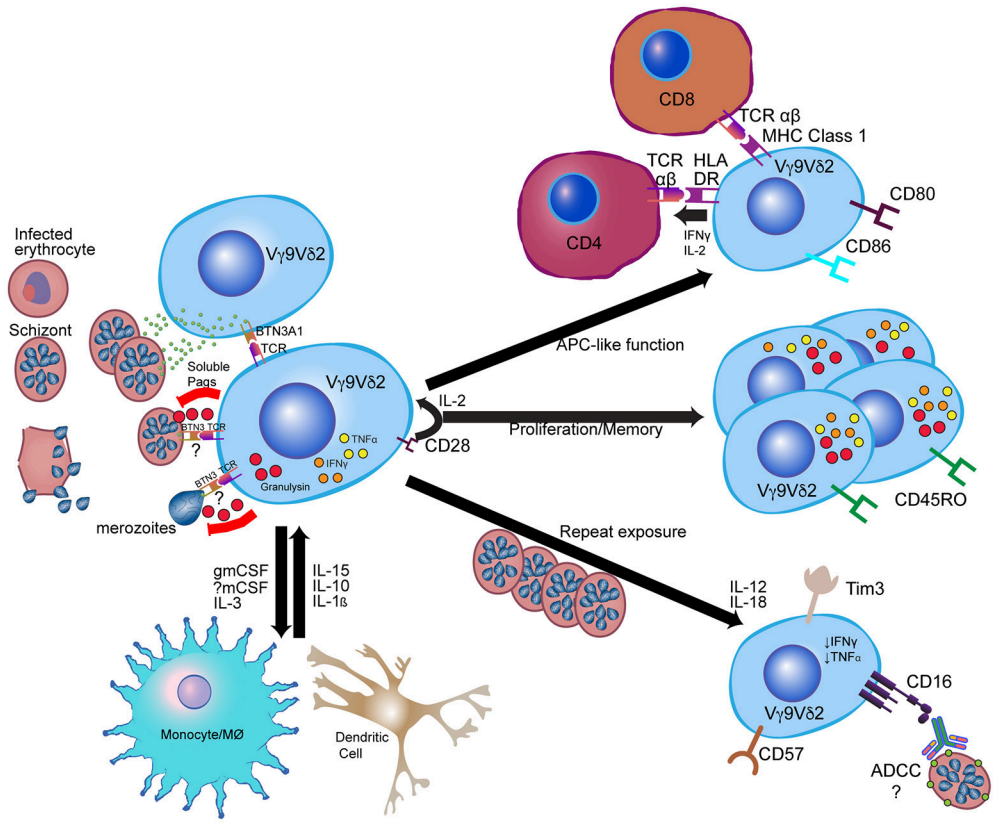

FIGURE 1 | Model for Vγ9Vδ2 γδ T cell response to *Plasmodium falciparum*.Vγ9Vδ2 γδ T cells recognize soluble phosphoantigens released from schizont stage parasites and potentially other iRBC stages. Though precise mechanisms of antigen presentation and recognition remain unclear, phosphoantigens are likely presented to Vγ9Vδ2 TCR via BTN3A1. Other signals, such as CD28 and IL-2 from CD4+ cells and IL-15 from myeloid cells, contribute to Vγ9Vδ2 proliferation and anti-parasitic activity, while Vγ9Vδ2 anti-parasitic activity is dependent on granulysin production. Following activation, Vγ9Vδ2 cells likely influence myeloid cell differentiation and activation through production of myeloid growth factors, and can themselves develop antigen-presenting cell (APC)-like functions such as activation of CD4+ T cells and cross-presentation of antigen to CD8+ T cells. After repeated parasite exposure, cells decrease production of pro-inflammatory cytokines and increase expression of CD16 and immunoregulatory molecules such as Tim-3. CD16 expression may enable alterative functions, such as antibody-dependent cellular cytotoxicity (ADCC). Immune cell images are adapted from the Reactome Icon Library (49).

γδ T cells expressing NK cell receptors form the primary source of IFNγ (33, 66, 85) whereas others have argued that γδ T cells are important producers of TNFα but not IFNγ (86). These differential results could be due to differences in timepoints, donors, parasite strains, surface markers used to differentiate cell populations, or immune cell activation conditions; further research, particularly in defining the impact of diversity in γδ T cell numbers and TCR repertoires on heterogeneity of responses, is needed. It is also possible that γδ T cells are required via an unknown mechanism for effective cytokine production by NK cells (86). Which cytokines are produced is likely determined by a combination of factors decided in cell development (e.g., expression of CD27) and epigenetic/transcriptional changes induced by environmental factors (87).

Several *in vitro* studies have provided further insight into the conditions required for γδ T cell effector functions; while IL-2 induces γδ T cell proliferation, for example, IL-15 is needed for anti-parasitic activity (48, 88, 89). Granulysin (but not perforin) released through cytotoxic granules also appears to be required for anti-parasitic activity (48, 76), which is supported by the existence of granulysin-expressing Vγ9Vδ2 cells in patients with malaria (48). Interestingly, Costa et al. showed that while both blood-stage parasites and extracellular merozoites activate Vγ9Vδ2 cells and initiate degranulation, only merozoites trigger anti-parasitic activity by these cells (48). Though the RBC membrane has been suggested to be resistant to granulysin (90), it is currently unclear which parasite stages can be targeted by activated γδ T cells and whether intracellular stages can be targeted via granulysin or other mechanisms.

γδ T CELL ROLES IN STIMULATING OTHER INNATE AND ADAPTIVE IMMUNE RESPONSES IN RESPONSE TO *PLASMODIUM*

In addition to roles in cytokine production and cytotoxicity, there is increasing evidence that γδ T cells can recruit and

TABLE 2 | Associations between γδ T cells and protection in experimental *Plasmodium* infection models in mice.

Species	Strain	Characteristic	Finding	References
Plasmodium yoelii	Py17X (PyNL)	Non-lethal	• γδ T cells not essential for clearance of blood-stage parasites but do contribute to control of liver stages	Tsuji et al. (50); McKenna et al. (51)
Plasmodium berghei	ANKA	Lethal	• Mice with γδ T cells depleted by monoclonal antibody are protected from cerebral malaria • γδ T cells affect CD8α+ dendritic cells in the liver, antigen-specific CD8+ T cell responses in the liver and spleen, and development of protective immunity	Yañez et al. (52); Zaidi et al. (46)
Plasmodium berghei	XAT	Non-lethal	• γδ T cells essential for parasite clearance • CD40 signaling between γδ T cells and dendritic cells contributes to control of parasitemia • γδ T cells contribute to humoral immunity	Inoue et al. (53); Inoue et al. (54)
Plasmodium chabaudi	AS	Non-lethal in C57BL/6 mice	• γδ T cells expand after infection and produce IFNγ • Mice deficient in γδ T cells have higher parasitemia • γδ T cells expanding later in infection protect against parasite recrudescence	Van der Hyde et al. (55); Langhorne et al. (6); Seixas and Langhorne (7); Weidanz et al. (8); Weidanz et al. (56); Mamedov et al. (9)

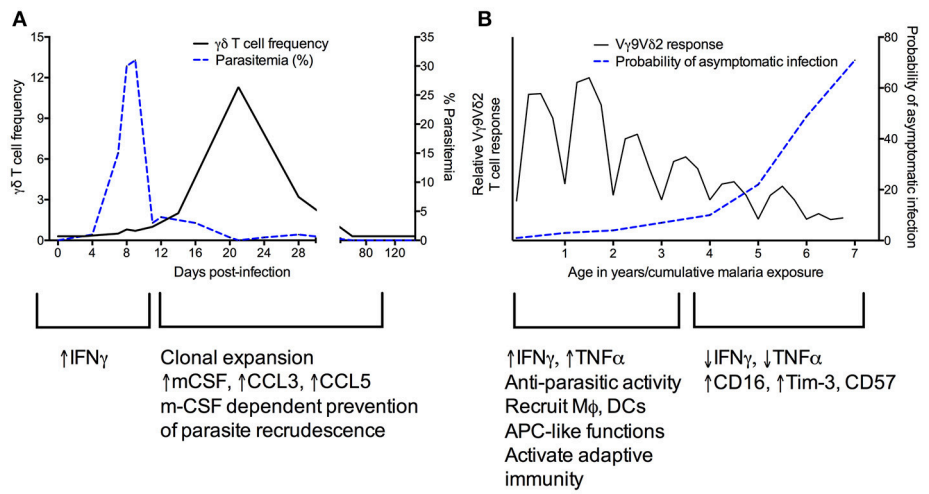

FIGURE 2 | Kinetics of γδ T cell responses during murine *Plasmodium chabaudi* **(A)** and recurrent human *Plasmodium falciparum* **(B)** infections. **(A)** [adapted from Figures 1A,E in Mamedov et al. (9)]. During the acute phase of *P. chabaudi* infection, γδ T cells primarily produce IFNγ. Proliferation of γδ T cells later in infection corresponds with increased production of cytokines such as M-CSF that influence the myeloid compartment, and in parallel, a decrease in parasitemia. **(B)** In human malaria, Vγ9Vδ2 T cells rapidly proliferate and produce pro-inflammatory cytokines during primary *P. falciparum* infection. These cells recruit and activate other immune cells and are able to kill parasites. After repeated infections, Vγ9Vδ2 T cells proliferate less and produce less IFNγ and TNFα while increasing expression of CD16 and regulatory markers such as Tim-3 (34, 35, 39). These changes correlate with an increased probability of asymptomatic infection.

stimulate other immune cells, and can adjust pro-inflammatory vs. regulatory effector functions depending on specific host or pathogen factors (for example, cytokines present in the microenvironment) (91, 92) (**Figures 1**, **2**). As described above, non-Vγ9Vδ2 cells were found to proliferate among individuals in a malaria-endemic region and produced the immunoregulatory cytokine IL-10, suggesting expansion of a non-cytotoxic, immunoregulatory population of γδ T cells (38). Furthermore, though γδ T cells have been known to function as antigen-presenting cells (APCs) in other contexts (93–96),

this phenomenon was recently demonstrated for the first time in malaria. Vγ9Vδ2 T cells from malaria-infected individuals more highly expressed antigen-presenting and costimulatory molecules, such as HLA-DR, CD80, and CD86, compared with healthy and nonmalarial febrile control subjects (**Figure 1**) (27). These cells were found to have APC-like functions in response to *in vitro* iRBC stimulation, including activating naive αβ T cell responses and cross-presenting protein to antigen-specific CD8+ T cells (27). Studies in humans have also shown that Vγ9Vδ2 T cells express the myeloid growth factors GM-CSF and IL-3 following *in vitro* stimulation with iRBCs (34). In mice, Mamedov et al. found that a specific γδ T cell clone (TRAVN-1+/V d6.3+) responsible for protection from *P. chabaudi* responded late in infection and prevented parasite recrudescence. Unlike IFNγ-producing γδ T cells responding earlier in infection (9), this clone produced macrophage-colony stimulating factor (M-CSF) and accessory cytokines that influence the myeloid compartment (i.e., CCL5, CCL3) (**Figure 2A**). Precise differences between IFNγ- and M-CSF-producing γδ T cells, including their direct vs. indirect roles in preventing parasite recrudescence, remain to be defined. Furthermore, whether malaria-induced expression of myeloid growth factors by γδ T cells directly or indirectly influences macrophage function, including epigenetic reprogramming of the myeloid compartment [potentially inducing trained immunity (97)], remains to be determined.

Just as γδ T cells could indirectly influence adaptive immunity through modulation of monocyte function, a parallel process could occur in dendritic cells (DCs). The previously mentioned PfSPZ vaccine trial and corresponding validation in the mouse model suggested that Vδ2+ γδ T cells may be essential for induction of dendritic cell and protective CD8+ responses (46). The absence of γδ T cells during murine vaccination led to dramatically reduced CD8α+ DCs in the liver, impaired antigen-specific CD8+ T cell responses in the liver and spleen, and resulting impaired development of protective immunity (46). These results highlight a possible role for γδ T cells in promoting the migration and/or proliferation of CD8α+ DCs and/or a requirement for cross-talk between γδ T cells and CD8α+ DCs in induction of downstream effector CD8+ T cell responses during PfSPZ vaccination (46). Interestingly, there was no impact of γδ T cell absence on production of antibody targeting the circumsporozoite protein (CSP); however, this does not preclude an effect on antibodies targeting other parasite antigens. Interestingly, γδ T cell expression of CD16, which is known to mediate antibody-dependent cellular cytotoxicity (ADCC) in CMV infection (24) is increased in children in malaria-endemic regions (34, 35), suggesting a potential role for γδ T cells in inciting antibody-mediated parasite killing (**Figure 1**). Finally, paralleling previous observations during influenza infection (98), recent work in the *P. berghei* mouse model demonstrated a role for γδ T cells in increasing levels of antigen-specific antibodies, Tfh cells, and germinal center B cells via expression of IL-21 and IFNγ early in infection (54). Altogether, evidence suggests that malaria-responsive γδ T cells are able to use diverse direct and indirect (via recruitment of monocytes, dendritic cells, and CD4+ cells) mechanisms to influence effector responses later in infection.

γδ T CELL MODULATION DURING RECURRENT INFECTION

New insights into changing γδ T cell functions during recurrent malaria infection bring up intriguing questions surrounding the relative importance of this modulation in natural immunity to malaria and whether this phenomenon represents a functional or dysfunctional response. Repeated *Pf* infection among Ugandan children was associated with reduced percentages of Vδ2+ γδ T cells, decreased pro-inflammatory cytokine production in response to malaria antigens, and increased expression of CD16 and CD57 and immunoregulatory genes such as HAVCR2 (encoding the inhibitory receptor Tim-3) (34) (**Figures 1**, **2B**). Importantly, though higher Vδ2+ pro-inflammatory cytokine production was associated with protection from subsequent infection, it was also associated with increased odds of having symptoms once infected (39). This suggests that Vδ2+ T cell dysfunction may represent a disease tolerance mechanism allowing for the development of "clinical immunity" to malaria—a decline in symptomatic infections and an increasing proportion of infections that are asymptomatic (34). Alternatively, it is also possible that with repeated exposure, γδ T cell responses gain alternative functional capabilities (i.e., CD16-mediated processes like ADCC).

Regarding mechanisms driving this dysfunction, co-engagement of CD46 and γδ TCR in cells stimulated by HMBPP has been shown to suppress production of IFNγ and TNFα, suggesting that CD46 could be involved in mediating γδ T cell regulation (99). Alternatively, there is evidence for reduced γδ T cell effector function in the setting of Tim-3 expression (37, 39), suggesting that repeated or chronic infection may induce Tim-3 mediated γδ T cell exhaustion, similar to what has been described in Th1 T cells (100, 101) and other innate cells (102). Consistent with this hypothesis, a study found that Tim-3 blockade in murine malaria improved T cell-mediated immunity (103). Schofield et al. recently described that Tim-3+ γδ T cells elevated in children living in malaria-endemic Papua New Guinea were independently associated with asymptomatic malaria infection, consistent with a role for Tim-3 mediated γδ T cell immunoregulation in minimizing symptoms due to malaria (37). Tim-3 expression by γδ T cells in this context was regulated by IL-12 and IL-18 (37). As IL-12/IL-18 can also induce IFNγ production, it will be important to assess the factors and timing differentiating this phenotype from Tim-3 expression, including the role of phosphoantigen.

Additional studies in neonates have highlighted that placental malaria affects the phenotype and repertoire of Vδ2+ lymphocytes in cord blood, potentially lowering the capacity for subsequent Vδ2+ responses to both malaria and other infectious diseases (32). Neonatal Vδ2+ T cells were recently shown to upregulate programmed death 1 (PD1), which when engaged by its ligand, PDL1, decreases TNFα production and degranulation by Vδ2+ cells (36). Intriguingly, PD1 expression by neonatal Vδ2 cells was inversely associated with promoter DNA methylation (36), suggesting a role for epigenetic programming in regulating inflammatory responses. Though much progress has been made in understanding the development of γδ T regulation,

further work is needed to more precisely define the underlying mechanisms and to reconcile *in vivo* observations with *in vitro* results showing an increased responsiveness to lysed *Pf* iRBCs after priming with intact iRBCs (85).

POTENTIAL FOR IMMUNOLOGICAL MEMORY IN MALARIA-RESPONSIVE γδ T CELLS

Altered γδ T cell function and upregulated immunoregulatory markers following repeated infection lead to exciting questions concerning the capacity of γδ T cells to develop immunological memory (104), whether similarly to canonical T cell memory or to innate memory. Though γδ T cells have historically been thought of as primarily innate-like, quick-responding cells, there is increasing evidence that these cells are also important at later timepoints during infection and have important adaptive-like functions. In response to CMV, γδ T cells developed a cytotoxic effector/memory phenotype, which in a secondary response led to a faster γδ T cell expansion and a better resolution of infection than the primary response (105). Similarly, distinct primary and recall/memory responses were observed in response to mycobacterial infection in macaques, in which γδ T cell ability to rapidly expand following BCG vaccination correlated with immunity to fatal tuberculosis (18). In controlled human malaria infections in malaria-naive adults, γδ T cells expand late after infection; elevated frequencies of cells expressing effector memory surface markers and enhanced responsiveness to *Pf* stimulation persisted for over 1 year (40). Furthermore, *in vitro* experiments demonstrated that the recall response of γδ T cells post-challenge was independent of other PBMCs (40).

In contrast to adaptive T cell memory, innate-like memory in γδ T cells is an intriguing alternative supported by evidence that monocytes can adapt secondary response to infection based on priming-induced epigenetic reprogramming (106, 107). A recent study has observed this phenomenon, termed "trained immunity", in monocytes stimulated *in vitro* with *Pf*-iRBCs or from children living in malaria-endemic regions (108). Preliminary evidence showing epigenetic reprogramming at the PD1 locus in neonatal Vγ9Vδ2 cells (36) suggests that a similar process could be responsible for the immunoregulatory phenotype seemingly acquired among γδ T cells after multiple infections (34, 39). Additional questions surrounding memory include the relative importance of adaptive-like vs. innate-like memory cells in protection from malaria, the localization of these cells, and the host and parasite factors influencing their development.

DIRECTIONS FOR FUTURE RESEARCH AND APPLICATION TO POTENTIAL THERAPEUTICS

Recent advances in our understanding of the role of γδ T cells in recurrent malaria infection and vaccination highlight numerous open questions and areas for future research **(Box 1)**. Whether γδ T cell activation occurs via cell-cell contact, soluble phosphoantigens released from rupturing schizonts (77) intact iRBCs (69), and/or other parasite stages, and how these cells are precisely able to target and kill *Pf*-iRBC, remains to be elucidated. Though Vγ9Vδ2 cells have been the most studied subset in human malaria, it will be important to determine the role of non-Vγ9Vδ2 cells in infection (38). In addition, evaluating the activation and role of γδ T cells in other important human Plasmodium infections, including *Plasmodium vivax* (109), will benefit eradication programs in diverse settings. Furthermore, a better understanding of human donor variability (66, 77) and parallels and differences between human and murine γδ T cell responses to malaria will enable better translation of observations between the experimental setting and the clinic.

Current work investigating plasticity of γδ T cells in malaria have exciting implications for our understanding of γδ T cell diversity and memory. For example, if Vγ9+ and Vγ9-Vδ2+ subsets serve distinct "innate-like" vs. "adaptive-like"

Box 1 | γδ T cells in response to malaria: outstanding questions and areas for future research.

γδ T cell activation and functional specialization.

- What are the precise mechanisms underlying Vγ9Vδ2 T cell activation in response to *Plasmodium falciparum*?
- What is the relative importance of intrinsic vs. extrinsic signals required for γδ T cell activation, proliferation, and anti-parasitic activity?
- How do Vγ9Vδ2 T cells target and kill malaria-infected red blood cells?
- What is the functional role of non-Vδ2+ T cell subsets in human malaria? How are these cells activated?
- What is the role of γδ T cell subsets in the response to non-*Plasmodium falciparum* strains? Given differences in mouse vs. human γδ T cell subsets, how should differences in responses between mouse and human studies be interpreted?

γδ T cell plasticity, memory, and altered function after repeated infection.

- How much diversity exists among malaria-responsive Vδ2+ cells (i.e., do separate "innate-like" and "adaptive-like" subsets coexist?)
- What malaria-induced cellular and microenvironmental cues drive γδ T cell differentiation and plasticity?
- Does dysfunction of Vγ9Vδ2 T cells following repeated malaria represent exhaustion, anergy, or, alternatively, immunologic memory and/or gain of function (e.g., ADCC, antigen presentation)?
- What mechanisms drive Vγ9Vδ2 T cell loss and dysfunction following repeated malaria? (i.e., altered cellular metabolism, epigenetic modifications?)
- Can altered γδ T cell function be reversed or made more functional?
- How do altered γδ T cell functions impact interactions with other immune cells (monocytes, dendritic cells, αβ T cells, B cells, T follicular helper cells)?
- What is the relative importance of innate-like "trained immunity" vs. canonical T cell memory in γδ T cell immunological memory? Does this balance differ in natural infection vs. vaccine-induced exposure?

Applications to novel therapeutics and vaccines.

- Can therapeutic approaches that prevent the development of Vγ9Vδ2 T cell dysfunction enhance parasite clearance?
- Can adjuvant approaches targeting γδ T cells (i.e., BCG, HMBPP, small molecules) influence the development of vaccine-induced malaria-specific humoral or αβ T cell responses?

functions in malaria as has been recently shown in acute CMV (16), this would significantly impact our understanding of differentiation and clonal expansion across age and repeated malaria exposure. Differential cytokine expression in the tissue microenvironment in cancer (91) can induce functional plasticity and differentiation of diverse γδ T cell subsets, but it remains to be determined whether cellular and/or environmental cues similarly drive differential cell fates in malaria. As repeated malaria has been shown to lead to dysfunction of Vγ9Vδ2 T cells, it will be important to assess whether this process represents exhaustion, anergy, or alternatively, immunologic memory and/or gain of function (e.g., ADCC). Mechanisms driving these alterations, including potential epigenetic and/or metabolic perturbations, should be examined (110). In addition, inflammation during recurrent malaria leads to suppression of functional memory B cells (MBC) (111) and expansion of "inferior" T follicular helper (Tfh) cells (112, 113) and "atypical" MBC exhibiting impaired proliferation, cytokine production and antibody secretion (114). Considering the overlapping timing of immunoregulatory phenotypes developing in γδ T cells and adaptive cells, as well as evidence for γδ T cells impacting germinal center reactions in murine malaria (54) and Tfh differentiation during influenza (98), future research should examine the impact of altered γδ T cell function on expansion of MBC and Tfh subsets and interactions with other immune cells.

Novel tools that reduce the immense global burden of malaria by improving natural immunity are urgently needed. Potential therapies involving γδ T cells could directly target γδ T cell responses or could include γδ T cell stimulation in an approach targeting antibody or T cell responses. The former approach could target activating or regulatory molecules or binding affinity/avidity to parasite antigens, induce intracellular accumulation of human Vγ9Vδ2 T cell agonists, and/or stimulate a particular γδ T cell function (e.g., ADCC via increasing CD16 expression). Clinically available compounds performing such functions led to accelerated clearance of *Yersinia pestis* and repair of inflamed tissue in non-human primates (115), and co-administration of Vγ9Vδ2 T cell agonists and IL-2 in cancer patients induced efficient activation of γδ T cells, and ultimately disease stabilization (116, 117). Interestingly, BCG has historically been used as treatment for bladder cancer, potentially by stimulating Vγ9Vδ2 T cells to more efficiently kill cancer cells (118, 119). Future studies investigating the role of BCG or other similar strategies may be useful to enhance responses to malaria and/or other infectious diseases. Therapeutic approaches to prevent γδ T cell dysfunction should also be explored. For example, experimental malaria infection given under chloroquine prophylaxis leads to long-term functional γδ T cell responses associated with protection against re-infection (40, 41), and preventing blood stage infection with highly effective antimalarial chemoprevention was also recently shown to prevent γδ T cell dysfunction in children (35). Drug development could also target inhibitory receptors involved in γδ T cell dysfunction (e.g., Tim-3) or epigenetic pathways involved in modulating plasticity (and possibly trained immunity) of γδ T cells (120).

Regarding approaches using adjuvant γδ T cell stimulation to augment vaccine-induced antibody or T cell responses, one group immunized non-human primates with a subunit vaccine for tuberculosis combined with phosphoantigen. The authors identified a robust γδ T cell response (including development of effector memory surface markers) following primary vaccination but anergy after subsequent boosts (121). In contrast, αβ T cells proliferated after boost vaccinations. These promising results indicate a need for further work aiming to maximize protective responses, whether by preventing γδ T cell anergy or optimizing timing of functional γδ and αβ T cell responses. As the balance between pro-inflammatory and anti-inflammatory responses changes drastically throughout malaria infection, the timing of any of these interventions would be essential to boosting responses without worsening pathology. Such interventions could also be useful for other infections that elicit chronic antigen exposure and/or an exhausted γδ T cell phenotype.

Successful therapies targeting γδ T cells for malaria will likely require a more thorough understanding of (1) functional differences between Vγ9Vδ2 T cell subpopulations, (2) migration and tissue infiltration of Vγ9Vδ2 T cells *in vivo*, (3) cellular interactions in the relevant microenvironment, (4) factors that influence γδ T cell differentiation and exhaustion, (5) γδ T cell detection of and response to metabolic changes in the host, and (6) factors that determine the balance between pro-inflammatory and immunoregulatory responses. The development of tools eliciting long-term, functional γδ T cell responses will be a much-needed addition to the campaign to eliminate and eradicate malaria.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Gamma/Delta T Cells and Their Role in Protection Against Malaria

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Whether and how γδT cells play a protective role in immunity against *Plasmodium* infection remain open questions. γδT cells expand in patients and mice infected with *Plasmodium* spp, and cytokine production and cytotoxic responses against blood-stage parasites are observed *in vitro.* Their expansion is associated with protective immunity induced by irradiated sporozoite immunization, and depletion of γδT cells in some mouse models of malaria excacerbates blood-stage infections. It is now clear that these cells can have many different functions, and data are emerging suggesting that in addition to having direct parasitocidal effects, they can regulate other immune cells during *Plasmodium* infections. Here we review some of the historic and more recent data on γδT cells, and in light of the new information on their potential protective roles we suggest that it is a good time to re-evaluate their activation requirements, specificity and function during malaria.

Keywords: gamma/delta T cells, malaria, activation, human, mice, skin, liver, red blood cells

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INTRODUCTION

Malaria is endemic in large parts of tropical and subtropical countries with high morbidity and mortality. After a period of decline in the number of cases each year, malaria incidence is on the rise again, partly because of increased resistance against drugs and insecticides. Effective vaccines for malaria are therefore urgently needed. Whole organism vaccines, such as those containing irradiated sporozoites are promising candidates, and could confer sterilizing immunity against *Plasmodium falciparum* (1, 2). The mechanisms of protective pre-erythrocytic immunity are not fully elucidated, but are commonly assumed to be mediated by antibodies and $CD8^+$ T cells (3). Recently, however, a subset of T cells carrying γδT cell receptors (TCRs) has been shown to associate with protection induced by irradiated sporozoites (4). This observation has sparked a renewed interest in the potential role of γδT cells in protective immunity and immunoregulation in malaria. Studies of γδT cells in malaria were first published nearly 30 years ago, and since then there has been substantial progress in understanding the biology of these cells. However, relatively little research has been done applying this more recent knowledge to the investigation of malaria immunity. Here we review some of the historical literature on γδT cells in malaria in both human studies and experimental models of malaria in the context of more recent findings on development, function and recognition of these cells in the hope that it spurs more widespread interest in their possible role in malaria.

γδT CELLS

Until recently, it was thought that γδT cells were simply innate immune T cells with limited or somewhat redundant functions. The current view is that these cells complement many different

players of the immune defense system (5), and, it is becoming clear that they are heterogeneous populations of cells with important unique roles in many infections, autoimmune diseases, allergies and in immunoregulation. To understand what they do in malaria, it is important to understand their complexity; location, functional capabilities, the antigens they recognize and how they are activated.

The development and tissue locations of different γδT cells are not directly comparable between humans and mice, and therefore care has to be taken when extrapolating from one to the other. In both cases, γδT cells are generated in the thymus from $CD4^-$ $CD8^-$ double negative (DN) progenitor cells, which commit to the αβ or γδT cell lineage depending on the type of V(D)J rearrangements and the strength of the pre-TCR signal (6, 7). In humans, the repertoire of Vδ and Vγ genes is much smaller than that for αβT cells (8), with Vδ1, Vδ2, and Vδ3 chains being the most frequently used Vδ gene segments. These can pair with one of the several functional Vγ gene segments; Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9, or Vγ11, although some combinations are more likely than others. In healthy human adults, the majority of γδT cells in peripheral blood are $V\gamma9V\delta2^+$ T cells, and typically represent between 1 and 10% of circulating lymphocytes. These cells can also be found as a minority in gut, liver and other epithelial tissues, whereas $V\delta1^+$ γδ cells are present in higher frequencies at these sites (9).

In mice, DN progenitors in the thymus give rise to temporal waves of discrete populations of γδT cell precursors that populate distinct anatomical sites (6, 7, 10, 11). The first waves of γδT cells arise during embryonic development and bear invariant TCRs. Cells bearing the $V\gamma5V\delta1^+$ TCR or dendritic epithelial T cells (DETC) emigrate to populate the skin epidermis, and $V\gamma6V\delta1^+$ T cells will inhabit the reproductive tract, oral mucosa, peritoneal cavity and some other tissues, such as liver, lung, intestinal lamina propria, dermis etc. A third wave, produced at around birth, is characterized by $V\gamma7V\delta4^+$ TCRs, and populates the small intestinal epithelium. Subsequently, $V\gamma1^+$ and $V\gamma4^+$ γδT cells leave the thymus and recirculate between peripheral blood and lymphoid tissues, such as the spleen. These $V\gamma1^+$ and $V\gamma4^+$ γδT cells are the only γδT cells that are produced throughout life. Thus, for both species, the final tissue distribution of γδT cell subsets is related to a greater or lesser extent by their TCR chains (12).

The preferential location of different γδT cell subsets is important for understanding their role in malaria, where encounters with *Plasmodium* in the vertebrate host can occur in many different sites; skin, liver, peripheral blood and lymphoid organs. While γδT cell TCRs are distinct in human and mouse, it seems that in both cases γδT cells in tissue sites are different from circulating γδT cells, and some functions may be conserved across the two species [reviewed in (12)].

γδT CELL RESPONSES IN HUMAN AND MOUSE *PLASMODIUM* INFECTIONS

The malaria parasite is present in different locations during its life cycle in the vertebrate host: trafficking sporozoites in the skin, within hepatocytes in the liver, and a replicative cycle of invasion into, and egress from erythrocytes in peripheral blood with circulation through lymphoid organs, particularly the spleen (**Figure 1**). Encounters with γδT cells can therefore be multiple, and we need to incorporate our knowledge of different populations of these cells when considering their role in malaria: their recognition specificities, their locations, their possible effector or regulatory functions and their "memory" status, all of which could influence the outcome of a malaria infection (**Table 1**).

γδT Cell Responses Against Sporozoites

Infection of humans with live sporozoites under chloroquine prophylaxis (28) or after immunization with irradiated sporozoites (1, 4) results in a long-lasting expansion of γδT cells with a "memory" phenotype. Although these cells are detected in peripheral blood, it is not known at which stage of the parasite life-cycle, or where, they were induced (skin, draining lymph nodes, infected hepatocytes or peripheral blood/spleen, see **Figure 1** and **Table 1**), whether they recognize antigens expressed uniquely at the sporozoite stage, or even whether they were activated by parasite antigen *per se*. That they express $V\gamma9^+V\delta2^+$ TCRs (1, 29) suggests that they may be circulating γδT cells with access to many tissues. In humans, their activation and effector site is difficult to establish. Although the subpopulations of mouse and human γδT cells are not directly equivalent, the similar preferential tissue locations, eg circulating Vγ4 and Vγ1 mouse γδT cells and $V\gamma9V\delta2^+$ human cells; tissue-resident $V\delta1^+$ cells in humans and $V\gamma5^+$ and $V\gamma6^+$ in mice, are such that one could pursue this in mouse models.

Whether and how γδT cells interact with sporozoites in the skin is unknown, but given their appearance after sporozoite immunization, this would be an area of research worth pursuing. Rapidly activated γδT cells could either have some direct effector function or recruit other effector cells to prevent further development of the infection. The use of mice that lack epidermal DETCs or dermal $V\gamma6^+$ γδT cells (50, 51), may be useful tools to determine the importance of skin-residing γδT cells in malaria.

The association of γδT cells with protection after irradiated sporozoite immunization (1, 4) and the demonstration of the protective effects of γδT cells in irradiated sporozoite vaccination in *P. yoelii* and *P. berghei* mouse models (4, 32, 33) also suggest that these cells have an important protective role in the liver. The views are that γδT cells act either as effector cells that operate in the absence of αβT cells, or as accessory cells for appropriate protective responses from other cells (4). With the differences in the experimental approaches and the heterogeneity of γδT cell functions now known, it is likely that γδT cells could be performing both functions in these experimental models. It will also be important to determine whether intrahepatic (possibly $V\gamma6^+$) or blood $V\gamma1^+$ or $V\gamma4^+$ γδT cells are playing the protective role (52). In Zaidi et al.'s mouse model, the $V\gamma4^+$ γδT cells do not appear to have a role in protection, while the nearest equivalent in humans, blood $V\gamma9V\delta2^+$ cells are found to associate with protection. We have some clues about γδT cells induced by pre-erythrocytic stages of *Plasmodium* but many questions remain: which γδT cells? Where are the cells activated?

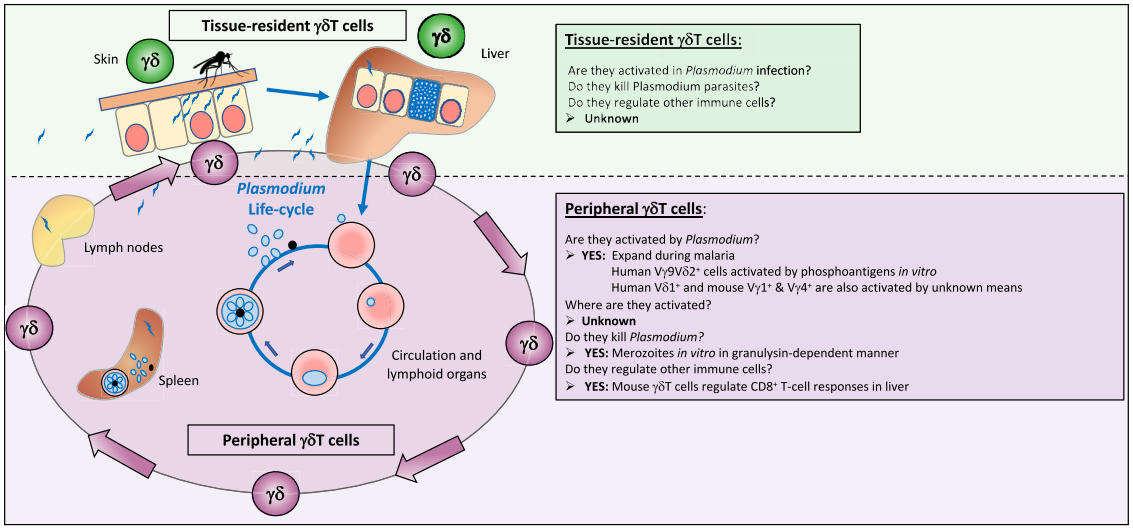

FIGURE 1 | γδT cells in malaria. Infected mosquitoes inject *Plasmodium* parasites in the form of sporozoites into the skin of a susceptible host from where they migrate to the liver to find an appropriate hepatocyte for invasion and replication. Some of these sporozoites will end up in lymphoid organs, such as spleen and lymph nodes as well. The parasite in hepatocytes undergoes rapid multiplication to form merozoites, which burst out of the infected cell and enter the blood circulation where they infect red blood cells and initiate multiple rounds of maturation and replication until the immune system manages to eliminate the parasites from the blood. During all these different steps—passage from skin to liver to blood—γδT cells present in the tissues (both tissue-resident and circulating γδT cells) could recognize parasites and become activated. Circulating γδT cells become activated during malaria, but nothing is known about where they are activated, whether in the skin, the liver or the lymphoid organs, and whether they really contribute to the antiparasite response during a natural infection. Tissue-resident γδT cells in the skin and liver could become activated, and protect against a new infection. Even less is known about responses of tissue-resident γδT cell subsets, the antigens they recognize, and whether they are able to kill sporozoites or infected hepatocytes.

What do they recognize? If they are accessory cells how are they functioning? If they are direct effector cells, what is their mechanism? The mouse models will be a good way to address these issues.

γδT Cell Responses Against Blood-Stages

The first observations of γδT cell responses to blood-stage malaria parasites were made more than 25 years ago, and showed that Vγ9Vδ2$^+$ γδT cells of malaria-naïve individuals proliferate in response to *P. falciparum*-infected red blood cells (iRBCs) *in vitro* (14, 16, 30, 31). Following this, it was demonstrated that Vγ9Vδ2$^+$ cells increase to up to 10–30% of total PBMC in *P. falciparum*- or *P. vivax*-infected adults with little or no previous exposure to malaria (14–18). More recently, this has also been shown in experimentally infected individuals (19). In regions of high malaria endemicity, or after multiple infections, healthy individuals already have a higher frequency of γδT cells than European populations (53), and there is no further peripheral expansion of Vγ9Vδ2$^+$ γδT cells on exposure to the parasite; however, there is a large increase in the proportion of Vδ1$^+$ cells in the PBMCs (20–24). The reasons for the relative expansion of Vδ1$^+$ cells is not understood but could be due to the retention of active Vδ2$^+$ cells in the spleen, thus changing the proportions in peripheral blood, or to the circulation of Vδ1$^+$ cells normally activated and residing in tissues, such as liver and skin.

γδT cells also increase in most of the rodent models of malaria studied. They are expanded in spleens of mice infected with different strains of *P. chabaudi, P. yoelii, and P. berghei* within 1–2 weeks of a blood-stage infection, depending on the mouse/parasite combination, reaching a peak at 3 weeks post-infection in non-lethal infections (13, 19, 34, 35), but with no expansion in a lethal *P. yoelii* infection (26).

The advantage of mouse models is that they can tell us whether the γδT cell response observed during blood-stage infection plays any protective role. In all the different infections studied—*P. yoelii* XNL,XL, *P. berghei* XAT, *P. chabaudi* AS, AJ, *P. chabaudi adami* K556A—mice without functioning γδT cells due to *in vivo* depletion with specific antibodies, or because of targeted deletion of the δ gene, show exacerbated acute parasitemias (although the increase is more pronounced with *P. yoelli* than with, eg, *P. chabaudi*) (19, 26, 27, 40, 41, 46–48), and in some cases this results in a lethal infection (26, 42, 48). In *P. chabaudi*, depletion of γδT cells additionally results in delayed clearance (27, 40, 41, 46, 47), or an increased magnitude of chronic parasitemias (19).

It is important to know which subpopulations of γδT cells in the mouse are responsible for the protective effect, as this could give us clues about where the γδT cells may have been activated as well as the nature of the inducing antigens. The γδT cell response to blood-stage *Plasmodium* in humans is dominated by cells bearing Vγ9Vδ2$^+$ TCR-chains, and thus one might predict that the nearest mouse counterparts are the blood/lymphoid circulating Vγ1$^+$ or Vγ4$^+$ γδT cells. However, the mouse response, at first glance, appears to be more heterogeneous. Although most reports show the circulating γδT cell bearing Vγ1$^+$, and/or Vγ4$^+$ TCRs to be expanded with different δ chains

TABLE 1 | γδT cells in malaria: human and mouse.

	Human	Mouse
KNOWN γδT CELL SUBSETS[a] EXPANDED DURING *Plasmodium* INFECTION IN		
• Skin (sporozoite entry) • Liver (liver-stage infection) • Peripheral blood & lymphoid organs (blood-stage infection)	• Not known • Not known • Vγ9Vδ2 (14–19), Vδ1 (20–24)	• Not known • γδT cells expanded (4); subset not known • Vγ1 (13, 19, 25), Vγ2 (25–27), Vγ4 (25)
γδT CELLS EXPANDED BY		
• Irradiated/Live sporozoites • Liver-stage parasites • Blood-stage parasites	• Yes (1, 4, 28, 29) • Not known • Yes—*in vitro* by *P. falciparum* (14, 16, 30, 31)	• Yes (4, 32, 33) • Not known • Yes—*in vivo*; variable dependent on *Plasmodium* spp. (13, 19, 26, 34, 35)
Antigen(s)	• Vγ9Vδ2: possibly phosphoantigens (36) • Vδ1: Not known	• Not known
Co-stimulation requirements	• CD28/CD80/86 (37), IL-2 (37), IL-15 (38)	• CD28/CD80/86 (37), IL-2 (34, 37, 39–42)
POTENTIAL EFFECTOR FUNCTIONS AGAINST		
• Sporozoites • Liver-stages • Blood-stages	• Not known • Not known • Vδ1, Vγ9Vδ2: degranulation and granulysin decrease *P. falciparum* replication *in vitro* (38, 43, 44) • IFNγ-production by Vγ9Vδ2 cells induced by *P. falciparum* iRBC *in vitro* (22, 28, 40, 43, 45)	• Not known • Not known • IFNγ-production during blood-stage infection (27)
PROTECTIVE AGAINST INFECTION BY		
• Sporozoites • Blood-stages	• Vγ9Vδ2 cell expansion associated with protection in irradiated sporozoite vaccination (1, 29) • Not known	• Yes - by recruitiment of CD8α+ dendritic cells which cross-present to effector CD8+ T cells (4) • Lack of γδT cells: variable effect on parasitemia depending on *Plasmodium* spp. (19, 27, 35, 40–42, 46–48). Subset unknown • Vγ1 γδT cells and M-CSF protect against chronic *P. chabaudi* (19)

[a] *γδT cell subset as determined by γ [mouse, Tonegawa 1986 nomenclature; (49)] or δ (human) T cell receptor chain expression.*

(13, 19, 25), there are also reports of $V\gamma2^+$ T cells (25, 27, 35). This could reflect the very different infections of *P. chabaudi*, *P. yoelii* and *P. berghei* strains in the mouse, and the different mouse strains used. However, some of the differences between different studies may be due to the confusing systems of nomenclature used for designating the γ chain of the mouse γδTCR (49, 54–56). Many of the earlier papers do not define clearly the nomenclature used. Given the contribution of mouse γδT cells to the control of blood-stage infections in mouse models, it would be well worth revisiting this, and reanalysing the γδT cell response in the different blood-stage infections.

All the blood-stage mouse infections described here differ in one major respect from natural infection, or sporozoite vaccination, in that they are not initiated via the bite of an infected mosquito. Not only does this mean that two key sites of potential γδT cell activation, skin and liver, are missing, but also the parasites from serial blood passage may differ in their transcriptional profile and in virulence (57, 58). The lack of the pre-erythrocytic stages of *Plasmodium* could influence location, specificity and dynamics of γδT cell activation. While the mouse γδT cell subsets are not direct counterparts of human, if we are to use the mouse model to elucidate mechanisms of γδT cell activation and protection, we should approximate as closely as possible to the mode of infection in humans.

γδT CELLS: ANTIGEN RECOGNITION AND ACTIVATION

Unlike αβT cells, antigen recognition by the γδTCR is not restricted to the classical major histocompatibility complex (MHC). Some γδT cell subsets do recognize members of the MHC superfamily or MHC-like molecules, such as CD1, other γδT cell subsets recognize full proteins or unique pathogen-associated molecular patterns (both of foreign and self-origin), whereas for other γδT cells, probably the vast majority of them, we do not have any idea of the type of antigen they recognize (5). The lack of diversity amongst V chain composition, especially in tissue-resident γδT cells, suggests that foreign antigen may not be the primary target of these cells, and suggests a role for γδT cells in lymphoid stress surveillance, perhaps with self-stress molecules representing the primary γδTCR-ligands (5). Which begs the question what do they recognize in a *Plasmodium* infection?

Human peripheral blood $V\gamma9V\delta2^+$ γδT cells recognize phosphoantigens, the most potent being (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), an intermediate in the alternative non-mevalonate pathway of isoprenoid biosynthesis. HMBPP is essential for the production of sterol-containing biomolecules including cholesterol, heme and steroid

hormones (5, 8). This pathway is used by *Plasmodium* spp. and other apicomplexa, as well as plants and bacteria, but not by higher eukaryotes, suggesting that these parasite products could stimulate Vγ9Vδ2$^+$ T cells without compromising self-tolerance. A soluble molecule with the same characteristics is produced by mature blood-stage *P. falciparum* (http://plasmodb.org; Gene ID: PF10_0221), and secreted during parasite egress (36). However, it has not been directly shown that HMBPP is responsible for Vγ9Vδ2$^+$ T cell activation in *Plasmodium* infection. Isopentenyl pyrophosphate (59), which can be produced by higher eukaryotes in the mevalonate pathway, is similar to but less potent than HMBPP in activating Vγ9Vδ2$^+$ T cells (5, 8), and could be another source of phosphoantigens during *Plasmodium* infection.

How Vγ9Vδ2$^+$ cells interact with phosphoantigens is not clear, as no direct contact with soluble/secreted HMBPP has been described. As the cells need cell contact for their activation, it is likely that an additional cell-surface molecule on the target is needed. Butyrophilins eg BTN3A or CD277, play an important role in the activation of Vγ9Vδ2$^+$ cells in response to phosphoantigens. One proposal is that BTN3A works as a cell surface antigen-presenting molecule. More recently it has also been proposed to work as an intracellular detector of phospoantigens that is capable of translocating to the cell surface to stimulate Vγ9Vδ2$^+$ T cells (60, 61). These findings suggest that Vγ9Vδ2$^+$ T cells may not need direct interaction with iRBCs for their activation. As we do not know where they recognize their specific antigens, and where they could carry out any effector functions, we can only speculate on the source of antigen in malaria.

There is no direct counterpart for Vγ9Vδ2$^+$ cells in the mouse, and there is no evidence that the circulating γδT cells of the mouse recognize phosphoantigens, therefore it is difficult to compare TCR specificities. Perhaps the best use of the mouse models would be to discover where these circulating γδT cells are activated and carry out their functional activities, rather than in a search for antigen specificity.

Human γδT cells expressing the Vδ1 chain and different γ chains, are abundantly present in tissues and normally form a minority in peripheral blood. Nevertheless, their frequency is increased in *Plasmodium* infections in areas where malaria is hyperendemic (20–24), and in other infections, such as HIV (62) and in the liver in HCV (59). The TCR of Vδ1$^+$ γδT cells has an oligoclonal repertoire distinct from that of circulating γδT cells (5, 63). These cells are highly enriched in epithelial tissues, can recognize a range of epithelial tumors, possibly through recognition of stress-induced MHC class I-related molecules MICA and MICB, can respond to autologous and/or endogenous phospholipids presented by CD1, and display TCR-driven clonal expansions in response to Cytomegalovirus (along with the minor Vδ3$^+$ and Vδ5$^+$ subsets), and possibly HIV as well as malaria (64, 65). This raises questions about why and how the Vδ1$^+$ T cell subset is expanded in Africans (20–22, 53). There is no evidence that they respond to *Plasmodium*-iRBCs *in vitro* (14), but of course there may be other activation requirements not provided in tissue culture, e.g., interleukin-2 (IL-2). We can only guess at the precise ligands that could activate these "epithelial" γδT cells in malaria. A strong possibility could be that they are recognizing stress-related molecules perhaps in the liver or skin as a result of pre-erythrocytic infection, or in the liver as a result of coping with chronic blood-stage infections. It is currently not known whether mouse DETCs and Vγ6Vδ1$^+$ are activated and expand during primary or repeated *Plasmodium* infections, or which antigens they would recognize in such a situation.

In addition to their TCR, γδT cells express other receptors as well, including toll-like receptors, CD16, CD226, natural killer receptors, and NKG2D (24, 66–68). Whether and which co-receptors are engaged in response to *Plasmodium* is an open question, and worthy of investigation as it may help explain how/why different γδT cells become activated in malaria.

γδT cells are rapidly activated and do not necessarily require a lymphoid environment. However, they do need costimulation for their proliferation and survival (37, 69). For human and mouse γδT cells responding to *Plasmodium,* costimulation is provided via interaction of CD28 on the γδT cell and CD80 and CD86 on the target/presenting cell. IL-2 appears to be a requirement for γδT cell activation to *Plasmodium*, either through an autocrine loop via TCR signaling (37, 41), or through the exogenous IL-2 provided by CD4$^+$ T cells (34, 39, 40, 42) and IL-15 can augment the IL-2-dependent response (38). A deeper understanding of the requirements for activation and maintenance of different γδT cells in malaria would be important for determining how to harness these cells for protective immunity.

FUNCTIONAL RESPONSES OF γδT CELLS IN MALARIA

It is becoming clear that γδT cells are more complex and varied in their immune roles than originally thought. Much of the work on function and roles of γδT cells has been carried out in mouse models, and although some aspects of γδT cells clearly vary between species, critical roles in early immune responses are often conserved. Common features of γδT cells include innate receptor expression, antigen presentation, cytotoxicity, and cytokine production (**Table 1**). However, the functional plasticity of Vγ9Vδ2$^+$ cells of humans observed *in vitro* or *ex vivo* (9) is not seen in mouse γδT cells, where cytokine profile is predetermined in the thymus and by their final tissue location (7, 9, 33, 70, 71).

Cytokine Production and Cytotoxicity

In humans and mice, rapidly activated blood/circulating/lymphoid γδT cells produce large amounts of interferon-γ (IFN-γ) after stimulation *in vitro* with *P. falciparum*-iRBCs (22, 28, 31, 43, 45) or during early blood-stage infection with *Plasmodium* (27). IFN-γ can mediate killing of parasites in infected liver cells (72), and more indirectly, activate phagocytes that can eliminate blood-stage parasites by antibody-dependent or -independent mechanisms (73). In the absence of αβT cells, γδT cells are required in some irradiated sporozoite immunization protocols to eliminate liver-stage parasites in mouse models (28, 32, 33, 45). However, it is not clear whether IFN-γ from γδT cells is crucial for these effector mechanisms in

malaria, as αβT cells and NK cells also produce this cytokine. On the other hand, loss of γδT cells in most mouse models of malaria does not compromise greatly the ability to remove acute blood-stage infections, although they may be important to control recrudescences (19, 26, 27, 32, 40, 41, 46–48).

Through their cytotoxic activity, it is conceivable that γδT cells can directly kill parasites (5). Both activated human Vδ2$^+$ and Vδ1$^+$ T cells degranulate when incubated with free merozoites, but not intraerythrocytic parasite stages, and can inhibit *P. falciparum* replication *in vitro* in a dose-dependent manner (43, 44, 74). This direct parasiticidal effect is dependent on granulysin rather than perforin, and requires contact or at least close proximity to target cells. Furthermore, patients infected with *P. falciparum* had elevated granulysin plasma levels and high numbers of granulysin-expressing Vγ9Vδ2$^+$ T cells, which degranulated when incubated in the presence of iRBCs (44). How effective these cytotoxic responses are *in vivo* remains debatable since merozoites only spend a very short time in the extracellular environment between egress and re-invasion. It might be more effective in tissues with a low blood flow, such as the red pulp of the spleen, where both γδT cells and mature iRBCs are highly prevalent and thus would have a higher chance of an encounter (75). Whether γδT cells can directly kill merozoites in mice, and whether this contributes to control of parasites has not yet been demonstrated. Perhaps the use of conditional knock-out mice in which the cytolytic machinery or IFN-γ-signaling has been specifically ablated in γδT cells or CD3DH mice which have reduced numbers of IFN-γ-producing γδT cells (71) would elucidate their roles more clearly.

There is now a wealth of literature about the involvement of different subpopulations of γδT cells in MHC class I presentation, regulation of other immune cells, and production of cytokines important for myeloid cell development. The most widely studied human γδT cells, Vγ9Vδ2$^+$ cells, have a wide variety of other functions including follicular helper-like, Th17-like, and Th2-like responses (9). Most of these aspects have not been explored in detail in malaria, but mouse models could offer good insights into how they contribute to the protective host response to *Plasmodium*. A recent example of an immunoregulatory function of γδT cells is the study by Zaidi et al. (4), where they have shown that γδT cells are important for the protective response induced by irradiated sporozoites, but it seems not as direct effectors. They are required for the development of an effective CD8$^+$ T cell response. In this immunization model, γδT cells were required for recruitment of cross-presenting CD8α$^+$ dendritic cells, necessary to activate effector CD8$^+$ T cells. How this is achieved is currently not known, but a recent paper on the interplay of γδT cells and myeloid cells may offer some clues. γδT cells producing macrophage-colony stimulating factor are important for controlling the chronic phase of *P. chabaudi* infections, suggesting that γδT cells are interacting with the myeloid cell compartment to control parasitemia (19, 76).

The functional capacities of epithelial γδT cells have not been investigated in great detail in malaria. As mouse skin and liver γδT cells produce IFN-γ and/or IL-17, and human skin Vδ1$^+$ cells, in addition to production of IFN-γ, can be cytotoxic, and these cells, when activated, recruit myeloid cells, and enhance phagocytosis (77, 78), such studies would be worthwhile.

γδT Cell "Memory" Responses

Long-term responses, or effective reactivation on second encounter with antigen requires some form of longevity of the cell population, either by constant re-stimulation, or through development of long-lived memory cells. Obviously for harnessing γδT cells in protective immune responses induced by vaccination it would be good to have an expanded population of "memory" cells that give an enhanced and more rapid response. The general view has been that γδT cells, although expressing TCRs encoded by somatically rearranged genes, are innate-like effectors that do not establish antigen-specific memory. It could also be argued that there is no need for the development of memory cells, as γδT cells have a relatively limited repertoire of TCRs which respond rapidly to the same set of antigens without the need for massive expansion, and this would happen on every exposure to appropriate antigens (79). Nevertheless, there are reports of adaptive-type memory responses of γδT cells. Human Vγ9Vδ2$^+$ T cell responses to phosphoantigens are increased by prior *Mycobacterium bovis* BCG vaccination (80). *In vivo*, there is a long term expansion of effector memory Vδ2$^-$ cells in human Cytomegalovirus infections (81) and enhanced "secondary" responses by Vγ9Vδ2$^+$ T cells in macaques infected with live Mycobacteria (82). Mouse "memory-like" Vγ6$^+$ γδT cells were maintained for more than 5 months in mesenteric lymph nodes after *Listeria monocytogenes* infection (83) and Vγ4$^+$ γδT cells have been found to persist in dermis and draining nodes for more than 3 months in a skin inflammation model (56, 84). Long-term elevation of γδT cells has been observed in peripheral blood of *P. falciparum*-exposed humans under chloroquine prophylaxis (28) or following irradiated sporozoite vaccination (1, 4), and in humans in malaria-endemic areas (53). Whether these contain true long-lived memory cells able to exist in the absence of antigens is not known. Such studies have not yet been carried out in either human or mouse *Plasmodium* infections.

SUMMARY

We have tantalizing evidence that γδT cells are important in the protective immune response to *Plasmodium*, particularly those induced by whole organism vaccination. It is also clear that we know little about which γδT cells are important, where and how they are activated and exactly how they contribute to protective immunity. Studies investigating γδT cells in the skin and liver, and especially mechanistic studies on the function of γδT cells in malaria are scarce or even lacking. Mouse models can help with several of these aspects, and now is the time to invest in this important part of the host response to *Plasmodium*.

AUTHOR CONTRIBUTIONS

KD and JL designed and drafted the manuscript.

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Metformin Promotes the Protection of Mice Infected With *Plasmodium yoelii* Independently of γδ T Cell Expansion

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Adaptive immune responses are critical for protection against infection with *Plasmodium* parasites. The metabolic state dramatically changes in T cells during activation and the memory phase. Recent findings suggest that metformin, a medication for treating type-II diabetes, enhances T-cell immune responses by modulating lymphocyte metabolism. In this study, we investigated whether metformin could enhance anti-malaria immunity. Mice were infected with *Plasmodium yoelii* and administered metformin. Levels of parasitemia were reduced in treated mice compared with those in untreated mice, starting at ∼2 weeks post-infection. The number of γδ T cells dramatically increased in the spleens of treated mice compared with that in untreated mice during the later phase of infection, while that of αβ T cells did not. The proportions of $V\gamma1^+$ and $V\gamma2^+$ γδ T cells increased, suggesting that activated cells were selectively expanded. However, these γδ T cells expressed inhibitory receptors and had severe defects in cytokine production, suggesting that they were in a state of exhaustion. Metformin was unable to rescue the cells from exhaustion at this stage. Depletion of γδ T cells with antibody treatment did not affect the reduction of parasitemia in metformin-treated mice, suggesting that the effect of metformin on the reduction of parasitemia was independent of γδ T cells.

Keywords: malaria, γδ T cell, clonal expansion, protection, metformin, metabolism

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INTRODUCTION

Malaria is caused by infection with *Plasmodium* parasites and is one of the most serious infectious diseases in the world. In endemic areas of tropical and subtropical countries, more than two million people suffer from malaria and ∼445,000 people died from the disease in 2016, according to a World Health Organization (WHO) malaria report (1). Strains of *Plasmodium falciparum* resistant to drugs, including artemisinin, are emerging and there is an immediate need for the development of effective vaccines. However, repeated infections and a prolonged amount of time are required for people living in endemic countries to gain natural resistance to malaria, and the memory response to *Plasmodium* antigens appears to be lost in the absence of repeated infections (2, 3).

It is important to define and understand the underlying mechanisms involved in the formation and maintenance of adaptive immune responses against *Plasmodium* infections to devise novel strategies for developing a malaria vaccine and to improve its effectiveness.

While antibody and $CD4^+$ T-cell responses are the primary effector mechanisms of protective immunity against blood-stage infection with *Plasmodium* parasites, several studies indicate that γδ T cells also participate in the immune response. Infection of humans with *P. falciparum* is associated with increased numbers of polyclonal γδ T cells in the peripheral blood (4, 5). In particular, γδ T cells expressing Vγ9 and Vδ2 are activated by the recognition of phosphorylated molecules of *P. falciparum,* resulting in cell proliferation and IFN-γ production (6, 7). Human γδ T cells inhibit replication and kill *P. falciparum* merozoites in a cell–cell contact-dependent manner, suggesting a protective role of γδ T cells against *Plasmodium* parasites (8). Another study showed that the reduction of $V\delta2^+$ γδ T cells, which respond to *P. falciparum,* in children repeatedly exposed to *Plasmodium* infection was associated with a reduced likelihood of symptoms upon subsequent infection with *P. falciparum*, suggesting a pathogenic role of human γδ T cells (9). In rodent malaria models, splenic γδ T cells increased 10-fold or more in C57BL/6 mice after infection with *P. chabaudi* and *P. yoelii,* while the increase of αβ T cells was limited (10–12). Unlike αβ T cells that expand during the early phase of infection, γδ T cell expansion occurs during the later period of infection (13–16). An increased abundance of γδ T cells is seen in β2-microglobulin knock-out mice, suggesting that the recognition of MHC class I is not required for the expansion of γδ T cells (11). γδ T cells produce cytokines such as IFN-γ and macrophage colony-stimulating factor (M-CSF) and have protective roles against *Plasmodium* infection (15, 16). Depletion of γδ T cells using a monoclonal antibody (mAb) resulted in persistent infection with the non-lethal *P. berghei* XAT strain, which is normally eliminated by the protective immune response (17). In this model of *P. berghei* XAT infection, γδ T cells expressed both CD40 ligand and interferon (IFN)-γ during the early phase of infection and enhanced the function of dendritic cells, thereby promoting protective immunity against parasites (15).

Recent studies revealed metabolic changes in T cells after their activation and during the generation of memory. Activated T cells switch the main pathway of adenosine triphosphate (ATP) generation from oxidative phosphorylation to glycolysis, which enables the generation of substrates required for synthesizing macromolecules such as nucleotides, proteins, and lipids, which promote rapid proliferation and effector function (18, 19). Metabolism in T cells is regulated by T-cell receptor (TCR) and cytokine-receptor signaling pathways involving Myc, hypoxia-inducible factor (HIF)-1a, and mammalian target of rapamycin (mTOR), which are crucial for regulating T cell activation and differentiation, and increasing or decreasing the metabolic output of cells in response to ligand stimulation (19). Adenosine monophosphate (AMP)-activated protein kinase (AMPK) senses the intracellular AMP/ATP ratio and induces a metabolic switch to promote ATP conservation by enhancing glucose uptake, fatty acid oxidation, mitochondrial biogenesis, and oxidative metabolism.

Metformin is widely used as an oral agent to treat patients with type-2 diabetes (20). Metformin is a derivative of the biguanide drugs, which were originally discovered as an antimalarial agent (21, 22). The antimalarial activities of the biguanide drugs were initially attributed to inhibition of the dihydrofolate reductase enzyme of the parasite, although additional mechanisms were subsequently proposed (23). Evidence suggests that the human mitochondrial respiratory-chain complex 1 is the target of metformin activity and that metformin binding to this target induces a drop in cellular ATP concentrations and increases the AMP:ATP ratio, resulting in AMPK activation (24). AMPK promotes oxidation of substrates in the mitochondria, thereby limiting the glycolytic capacity of cells (25). Recent studies suggest that metformin affects immune responses in mouse models of autoimmune disease and cancer. In a model of experimental autoimmune encephalomyelitis, treating mice with metformin resulted in reduced production of proinflammatory cytokines by T cells and slowed disease progression (26). In a cancer model, metformin enhanced the generation of memory $CD8^+$ T cells and helped to improve resistance against cancer (27). Metformin protected $CD8^+$ tumor-infiltrating T cells (TILs) from apoptosis and exhaustion, improved the function of TILs in producing multiple cytokines, and helped the shift from central memory to effector-memory T cell phenotypes (28). In the $CD4^+$ T cell compartment, metformin treatment increased the production of regulatory T cells (Tregs) (29). However, in the tumor microenvironment, metformin reduced tumor-infiltrating Tregs and the expression of effector molecules that act on tumor-infiltrating Tregs, which allowed the enhancement of sustained anti-tumor immunity (30). Taken together, these results suggest that metformin acts on T cells in various manners, depending on the T cell subset and on the environmental context in which they are activated.

In this study, we investigated the effect of metformin on the immune response against blood-stage infection with rodent *Plasmodium* parasites. We used *P. yoelii* strains, since these parasites are often used for studies of the regulation of T cell immune responses during the blood-stage of *Plasmodium* infection (31, 32). As expected, metformin inhibited parasitemia levels in mice infected with *Plasmodium* parasites. Unexpectedly, however, metformin treatment enhanced the increase of γδT cells during the later phase of infection with *P. yoelii* 17XNL, but not that of αβT cells. However, parasite clearance was not directly linked to the increase in γδT cells.

Abbreviations: 2NBDG, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose; 7-AAD, 7-amino-actinomycin D; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; APC, allophycocyanin; ATP, adenosine triphosphate; B6, C57BL/6; BrdU, bromodeoxyuridine; Cy7, cyanine 7; FITC, fluorescein isothiocyanate; HIF, hypoxia-inducible factor; IFN, interferon; mAb, monoclonal antibody; M-CSF, macrophage colony-stimulating factor; MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; ns, not significant; PE, phycoerythrin; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean; TCR, T-cell receptor; TIL, tumor-infiltrating T cell; TNF, tumor necrosis factor; Treg, regulatory T cell; WHO, World Health Organization.

MATERIALS AND METHODS

Mice, Parasites, and Metformin

C57BL/6 (B6) mice were purchased from Japan SLC (Hamamatsu, Japan). Mice were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University, and female mice were used at of 8–14 weeks of age. For infection experiments, a frozen stock of *P. yoelii* 17XL and *P. yoelii* 17XNL was thawed, washed, and inoculated intraperitoneally into B6 mice. When the parasitemia level reached 5–20%, the experimental mice were intraperitoneally infected with 10^4 red blood cells infected with *P. yoelii* 17XNL or *P. yoelii* 17XL in 500 μL PBS. Parasitemia was monitored by microscopic examination of standard blood smears. For storage, infected blood samples were mixed with the same volume of Dulbecco's modified Eagle's medium containing 20% dimethyl sulfoxide and 10% fetal calf serum, and frozen at −80°C or in liquid nitrogen. For metformin treatment, mice were provided *ad libitum* with water containing 5 mg/ml metformin (Nakarai Tesque, Kyoto, Japan or Tokyo Chemical Industry, Tokyo, Japan) starting on the day of the infection, unless otherwise indicated. To deplete γδ T cells *in vivo*, the mice were inoculated intraperitoneally with the anti-TCRγδ mAb GL3 (500 μg/mouse) on day −1, 0, or 1 relative to infection and then twice a week after infection, as reported previously (15). A hybridoma cell line producing anti-TCRγδ mAb (GL3) (33) was provided by Dr. F. Kobayashi (Kyorin University, Tokyo, Japan). Cells were cultured using a BD CELLine Flask (BD Biosciences), and mAb was purified from the culture supernatant using HiTrap Protein G columns (GE Health Care, Pittsburg, PA, USA). The animal experiments reported herein were conducted in accordance with the recommendations of the guidelines for Animal Experimentation, Nagasaki University. The protocol was approved by the Institutional Animal Care and Use Committee of Nagasaki University.

Flow Cytometry

Single cell suspensions of the spleen, inguinal lymph nodes, thymus, and bone marrow were pre-incubated with anti-CD16/CD32 mAb (2.4G2) for 10 min and then stained for 30 min with phycoerythrin (PE)-cyanine 7 (Cy7)-, fluorescein isothiocyanate (FITC)-, or BV510-conjugated anti-CD3 (145-2C11); FITC-conjugated anti-TCRβ (H57–597); PE- or allophycocyanin (APC)-conjugated anti-TCRγδ (GL3); APC-Cy7-conjugated anti-CD8 (53–6.7); BV711-conjugated anti-CD4 (RM4-5); PE-conjugated anti-TCR Vγ1(2.11); PE-conjugated anti-TCR Vγ2 (UC3-10A6); APC-conjugated anti-TCR Vγ3 (536); PE-conjugated anti-TCR Vδ4 (GL2); PE-conjugated anti-TCR Vδ6.3/2 (8F4H7B7); FITC-conjugated anti-B220 (RA3-6B2); APC-conjugated anti-CD27 (LG.3A10); FITC-conjugated anti-CD44 (IM7); PE-conjugated anti-CD69 (H1.2F3); FITC-conjugated anti-CD25 (PC61); APC-conjugated anti-TIM-3 (RMT-23); APC-conjugated anti-LAG-3 (C9B7W); APC-conjugated anti-PD-1 (RMP1-30); APC-conjugated anti-KLRG1 (2F1/KLRG1) mAbs; or biotin-conjugated anti-CD62L mAb (MEL-14) plus PE-conjugated streptavidin. Cells were stained with PE-conjugated anti-NKG2D (CX5) and FITC-conjugated anti-FCγRIII/II (2.4G2) without an anti-CD16/CD32 mAb. The antibodies were purchased from eBioscience (San Diego, CA, USA), BD Biosciences (San Jose, CA, USA), TONBO Biosciences (San Diego, CA, USA), or BioLegend (San Diego, CA, USA). To exclude dead cells from the analysis, 7-amino-actinomycin D (7-AAD; eBioscience) was added.

Analysis of cellular metabolism was performed as described previously (34). Briefly, splenocytes were pulsed with 50 μM 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2NBDG; Molecular Probes) for 30 min at 37°C and then stained for surface molecules in the presence of 20 nM MitoTracker Deep Red FM (MitoFM; Invitrogen, Carlsbad, CA, USA) for 30 min at 4°C. Samples were analyzed using a BD FACSCanto II or FACS Fortessa instrument (BD Biosciences). The data were analyzed using FlowJo software v10.2 (TreeStar, Ashland, OR). The numbers of spleen cells in each subset were determined by multiplying the total number of spleen cells by the percentage in each subset.

Intracellular Staining

To detect phosphorylated proteins, cells were stained with an APC-conjugated anti-TCRγδ mAb, fixed using a Phosflow Kit (BD Biosciences), and stained with a FITC-conjugated anti-CD3 mAb and PE-conjugated phospho-S6 (ser235/Ser236) antibody (eBioscience), a PE-conjugated phospho-mTOR (Ser2448) antibody (eBioscience), or a phospho-AMPK alpha 1 (phosphor T183) + AMPK alpha 2 (phosphor T172) antibody (Abcam, Cambridge, MA USA) plus a PE-conjugated anti-rabbit IgG antibody. To label proliferating cells, mice were intraperitoneally administered 1.4 mg bromodeoxyuridine (BrdU; BD Biosciences) 16 h prior to analysis. After staining cell-surface molecules, splenocytes were fixed, permeabilized, incubated with DNase, and intracellularly stained with an FITC-conjugated anti-BrdU mAb (3D4) (BioLegend), as described previously (35). To analyze cell death, cells were stained with annexin V (BioLegend) in annexin V-binding buffer (10 mM HEPES, 2.5 mM $CaCl_2$, 0.14 M NaCl, pH 7.5) for 30 min in accordance with the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO, USA) and were then stained with 7-AAD for 10 min.

For cytokine assays, splenocytes (1×10^6/well) were stimulated in 24-well plates with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 μM) for 6 h in RPMI 1640 medium, supplemented with non-essential amino acids, glutamine, sodium pyruvate, antibiotics, 10% fetal calf serum, 2-mercaptoethanol (5×10^{-5} M), and monensin. After pre-incubation with the anti-CD16/CD32 antibody 2.4G2, cells were stained with APC-conjugated anti-TCRγδ and BV510-conjugated anti-CD3 mAb; fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences); and stained with PE-Cy7-conjugated anti-IFN-γ (XMG1.2) (BioLegend), PE-conjugated anti-tumor necrosis factor (TNF)α (MP6-XT22) (eBioscience), PE-Cy7-conjugated anti-IL-2 (JES6-5H4) (eBioscience), or PE-conjugated anti-granzyme B (NGZB) (eBioscience) mAbs and then analyzed using a BD LSRFortessa X-20 instrument (BD Biosciences).

Immunohistochemistry

Spleen tissues were prepared for immunohistochemistry as previously described (36). Briefly, freshly frozen spleens were embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan), cut into 5-μm-thick sections using a cryomicrotome, and fixed in acetone for 15 min at room temperature. Samples were blocked using Blocking One Histo (Nacalai, Kyoto, Japan) for 1 h in a humidified chamber at room temperature. Sections were stained overnight at 4°C with PE-conjugated anti-TCRγδ and FITC-conjugated anti-TCRβ mAbs (all from BioLegend) and mounted in DAKO fluorescent mounting medium (Agilent Tech, Santa Clara, CA, USA). Images were acquired by fluorescence microscopy (Olympus, Tokyo, Japan) and merged using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

The data are presented in bar graphs as the mean ± the standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism (GraphPad Software). Comparison of two independent groups was performed using the unpaired *t*-test with Welch's correction. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. ns, not significant. Survival curves were compared using a log-rank (Mantel cox) test.

RESULTS

Metformin Promoted Clearance of Plasmodium Parasites

To study the effect of metformin on *Plasmodium* infection, mice were infected with *P. yoelii* 17XL or *P. yoelii* 17XNL and given metformin in their drinking water, starting on the day of infection. For mice infected with *P. yoelii* 17XL (**Figure 1A**) or *P. yoelii* 17XNL (**Figure 1B**), the levels of parasitemia increased similarly in both treated and untreated groups for ∼2 weeks post-infection. Lethal and non-lethal phenotypes of *P. yoelii* 17XL and *P. yoelii* 17XNL, respectively, are not always stable between experiments (37). Under our experimental conditions, some B6 mice survived *P. yoelii* 17XL infection, and *P. yoelii* 17XNL infection was lethal in some of the infected B6 mice. In *P. yoelii* 17XL-infected mice, nearly half of the metformin-treated and untreated mice died ∼10 days after infection. Among the surviving mice, the metformin-treated mice showed lower parasitemia levels and cleared the parasites earlier than untreated mice. In *P. yoelii* 17XNL-infected mice, the parasitemia levels were lower after metformin treatment, while no significant difference was observed in survival between the metformin-treated and untreated mice. We used the *P. yoelii* 17XNL-infection model in further experiments, since all mice survived at least 20 days using this model. This effect of metformin on *P. yoelii* 17XNL parasitemia was also observed when the mice were given metformin starting at 7 d post infection (**Figure 1C**). These results suggested that metformin promoted the clearance of the *Plasmodium* parasites in mice. In further study, we continuously treated B6 mice with metformin starting at day 0 of infection with *P. yoelii* 17XNL, since the level of parasitemia was stably reduced on day 18 in this model.

Increased γδ T Cells in Metformin-Treated Mice

Since metformin was previously found to improve the function of TILs (28), we examined the T-cell populations in the spleens of mice during *P. yoelii* 17XNL-infection using flow cytometry (**Figure 2**, **Figure S1**). The number of T cells ($CD3^+$ cells) increased in the first 6 days, decreased dramatically over the next 6 days, and then slightly increased during the following 6 days of infection in both metformin-treated and untreated mice (**Figure 2B**). This increase was not observed in mice treated with metformin alone for 18 days without *Plasmodium* infection (**Figure S2**). Consistent with a previous report, the proportion of γδ T cells among $CD3^+$ T cells increased by 18 d post-infection (**Figures 2A,B**) (15). However, the increase of γδ T cells was much higher in metformin-treated mice than in un-treated mice and reached >30% of the $CD3^+$ cells (**Figure 2A**). The number of γδ T cells in the spleen was stable during the initial 12 d of infection and increased between 12 and 18 d post-infection (**Figure 2B**). However, in the αβT cell fraction, both $CD4^+$ and $CD8^+$ T cells increased during the initial 6 d of infection and decreased thereafter (**Figure 2B**). No significant difference was found in the number of αβ T cells between the metformin-treated and untreated mice at 18 d post-infection. The increase in the number of γδ T cells occurred in the spleens of the metformin-treated mice, but not in the lymph nodes, bone marrow, or thymus (**Figure 3A** and **Figure S1**). No significant difference in B cell number occurred between metformin-treated and untreated mice (**Figure S3A**). The number of conventional dendritic cells was slightly higher in metformin-treated mice 12 days after infection with *P. yoelii* 17XNL. We did not find significant differences in serum levels of anti-parasite antibodies between metformin-treated and untreated mice, except for IgG3 levels 12 days after infection (**Figure S3B**).

Spleen sections were subjected to immunohistochemical analysis to determine the distribution of γδ T cells in the spleens (**Figure 3B**). γδ T cells were scattered in an area distinct from αβ T cells, suggesting that they were localized primarily in the red pulp of the spleen, consistent with a previous report (15). We then examined the frequencies of Vγ and Vδ usages in the increased γδ T cells (**Figure 3C** and **Figure S1**). The number of γδ T cells expressing Vγ1 or Vγ2 and those expressing Vδ chains, other than Vδ4 or Vδ6, significantly increased in metformin-treated mice. These cells showed a tendency to be increased even in metformin-untreated mice by infection with *P. yoelii* 17XNL, although the difference was not statistically significant. Therefore, we think that these γδ T cells were activated via the recognition of *Plasmodium* antigens in *P. yoelii* 17XNL-infected mice, and their expansion was enhanced by metformin treatment.

The increase in the number of γδ T cells induced by metformin treatment may have been due to either enhanced proliferation or to a reduction in cell death. Proliferation was evaluated using BrdU incorporation since γδ T cells in the infected mice were nearly 100% $Ki67^+$ (data not shown).

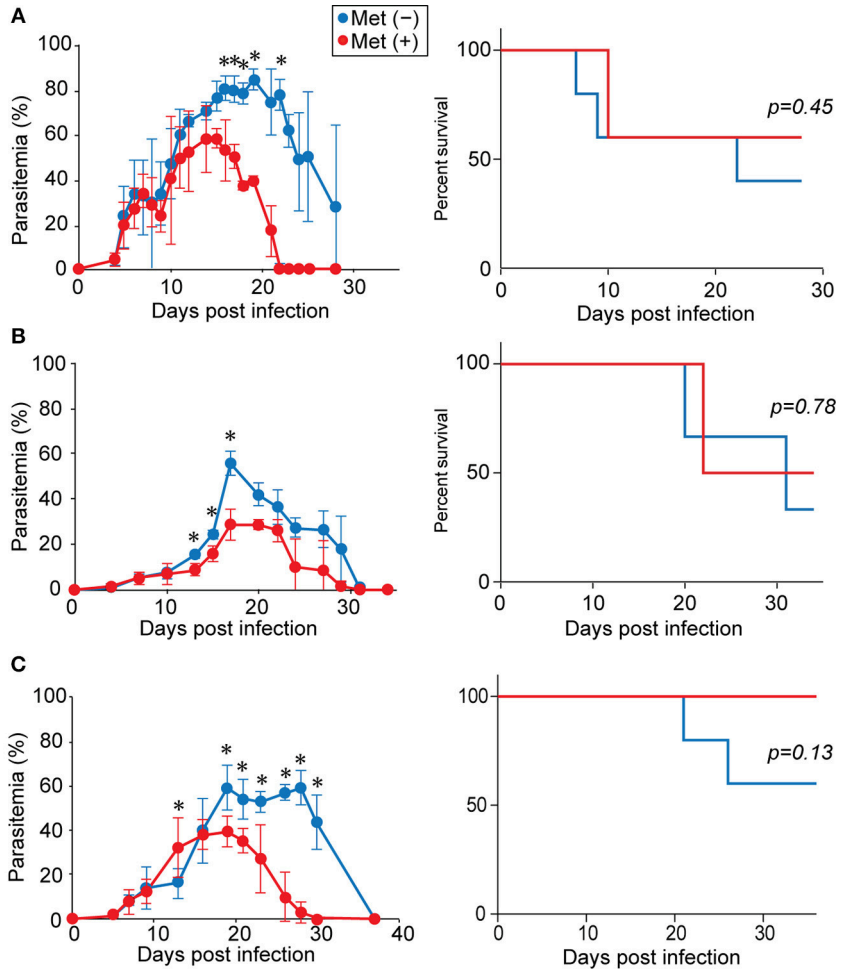

FIGURE 1 | Metformin reduced parasitemia and promoted the clearance of *Plasmodium* parasites. B6 mice were infected with *P. yoelii* 17XL **(A)** or *P. yoelii* 17XNL **(B)** and received metformin (Met^+, red line) or not (Met^-, blue line) in their drinking water, starting from the day of infection. Alternatively, B6 mice were infected with *P. yoelii* 17XNL and either did (red line) or did not (blue line) receive metformin in their drinking water, starting at 7 d post-infection **(C)**. The levels of parasitemia were monitored every few days. The data shown represent two **(A)**, three **(B)**, or one **(C)** experiment with 5 mice/group in each experiment. Statistical significance in the differences in parasitemia was assessed between the metformin-treated and untreated mice using the unpaired *t*-test with Welch's correction ($^*p < 0.05$). Survival curves were compared using a log-rank (Mantel cox) test.

Although the increase was not statistically significant, the proportion of $BrdU^+$ γδ T cells in metformin-treated mice tended to be higher than that in untreated control mice, while αβ T cells in the same cell preparation showed no difference (**Figure 4A** and **Figure S1**). The proportions of surviving $7AAD^-$ annexin-V^- γδ T cells were not different between the metformin-treated and untreated groups of mice (**Figure 4B**). Taken together, our findings suggest that metformin enhanced the proliferation of γδ T cells in *P. yoelii* 17XNL-infected mice.

Activation and Function of γδT Cells in Metformin-Treated Mice

We examined the phenotypes of γδ T cells in metformin-treated and untreated mice at 18 d post-infection with *P. yoelii* 17XNL (**Figures 5A–C** and **Figure S1**). The proportion of γδ T cells with an effector phenotype ($CD62L^{lo}$ $CD44^{hi}$) in metformin-treated mice was lower than that in untreated mice, while the proportion of γδ T cells with a central memory-phenotype ($CD62L^{hi}$ $CD44^{hi}$) was not significantly different between the metformin-treated and untreated groups of mice. In the αβ T cell compartment, however, $CD62L^{hi}$ $CD44^{hi}$ T cells were decreased in metformin-treated mice, while $CD62L^{lo}$ $CD44^{hi}$ T cells were not significantly affected (**Figure S4**). These changes were not observed in uninfected, metformin-treated mice (**Figures S2B,C**). B220 expression was upregulated on splenic γδ T cells in *P. yoelii*-infected mice, which was consistent with previously reported findings for hepatic γδ T cells (38), but B220 expression was not affected by metformin treatment. The proportions of γδ T cells expressing CD27, which is expressed on IFN-γ-producing γδ T cells (39), as well as the activation markers CD25, CD69, FCγR, and NKG2D (40–42) were not

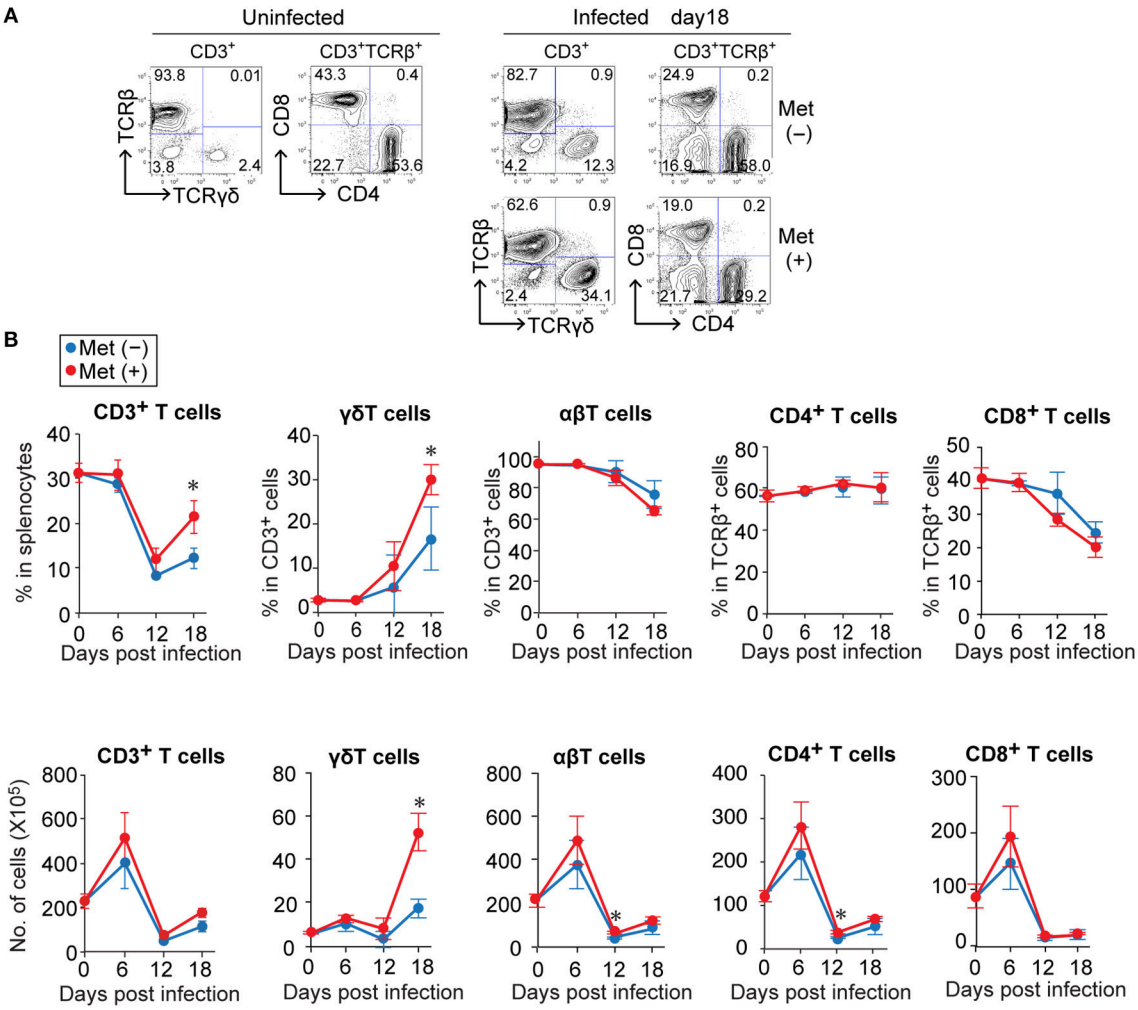

FIGURE 2 | The relative increase of γδ T cells in *Plasmodium yoelii* 17XNL-infected mice during the late stage of infection was greater for metformin-treated mice. B6 mice were infected with *P. yoelii* 17XNL and given metformin (Met^+) or not (Met^-) in their drinking water, starting from the day of infection. Splenocytes from uninfected and infected mice were stained for CD3, TCRβ, TCRγδ, CD4, and CD8 expression before (0 d) and at 6, 12, and 18 d post-infection, and were analyzed by flow cytometry. **(A)** Representative data for $CD3^+$-gated cells and $CD3^+TCR\beta^+$-gated cells before (uninfected) and 18 days after infection are shown. The number in each quadrant represents the proportion of the indicated cell populations. **(B)** The proportions (upper panel) and absolute numbers (lower panel) of T cell subpopulations in the spleens of mice infected with *P. yoelii* 17XNL at 0, 6, 12, and 18 d post-infection. The numbers were calculated by multiplying the total number of splenocytes by the relative proportion of each subpopulation. Each group was comprised of three mice. The data shown represent two independent experiments with similar results. Statistical significance was assessed using the unpaired *t*-test with Welch's correction (*$p < 0.05$).

affected by metformin treatment (**Figures 5A,B**). The expression of inhibitory molecules including KLRG1 that possess an ITIM motif in the cytoplasmic domain and has inhibitory function in T cells (43) was also evaluated. The γδ T cells expressed similar levels of LAG-3, PD-1, TIM-3, and KLRG1 in both metformin-treated and untreated mice (**Figures 5D,E**). Taken together, the expression of some activation markers on γδ T cells was reduced in mice treated with metformin, while the expression of inhibitory receptors was not affected.

To examine the effect of metformin on γδ T cell function, we evaluated the production of cytokines and granzyme B in γδ T cells at 6, 12, and 18 d post-infection with *P. yoelii* 17XNL (**Figure 6** and **Figure S1**). Prior to infection, 26.6 ± 11.8% and 24.4 ± 3.2% of the γδ T cells in the spleen could produce IFN-γ and TNF-α, respectively. The proportion of IFN-γ-producing γδ T cells increased at 6 d post-infection and then decreased thereafter, while that of TNF-α-producing γδ T cells continued to decrease during the infection. The γδ T cells able to produce granzyme B were not present before infection, but their numbers increased after infection. We did not detect any significant differences in the proportions of γδ T cells capable of producing cytokines and granzyme B in metformin-treated and untreated mice during the course of *Plasmodium* infection. In the αβ T cell compartment, reductions in IFN-γ- and TNF-α-producing $CD4^+$ and $CD8^+$ T cells were observed after 6 days of *P. yoelii* 17XNL infection. Unlike γδ T cells, production

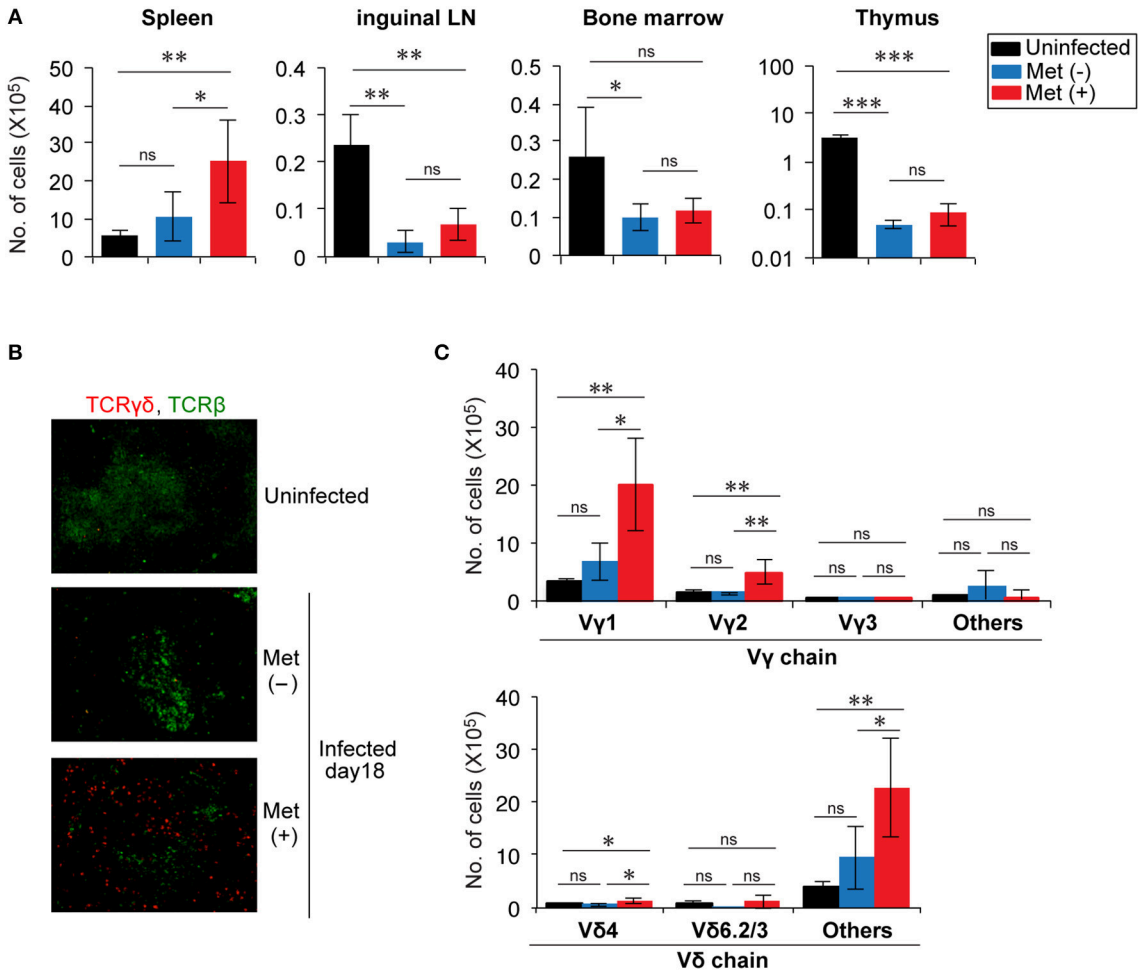

FIGURE 3 | Expansion of Vγ1$^+$ and Vγ2$^+$ T cells in the splenic red pulp of *Plasmodium yoelii* 17XNL-infected mice treated with metformin. **(A)** B6 mice were infected with *P. yoelii* 17XNL and given metformin (Met$^+$) or not (Met$^-$), as indicated. At 18 d post-infection, the numbers of γδ T cells in the spleens, inguinal lymph nodes, bone marrow, and thymus from uninfected and infected mice were determined as described in the legend of **Figure 2**. The data shown represent two independent experiments (3–4 mice/group) with similar results. Statistical significance was assessed by one-way ANOVA with Tukey's *post-hoc* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(B)** Spleen sections from uninfected and *P. yoelii* 17XNL-infected mice were stained with anti-TCRγδ (red) and anti-TCRβ (green) mAbs. **(C)** At 18 d post-infection, splenocytes from infected and uninfected mice were stained for CD3, TCRγδ, and for Vγ or Vδ, and analyzed by flow cytometry. The numbers of γδ T cells expressing the particular Vγ or Vδ chains are shown. The data shown represent two independent experiments (3–4 mice/group) with similar results. Statistical significance was assessed using one-way ANOVA with Tukey's *post-hoc* test (*$p < 0.05$, **$p < 0.01$, ns, not significant).

of granzyme B by CD8$^+$ αβ T cells was also reduced at 6 days post-infection (**Figure S5A**). Serum IFN-γ levels peaked at 12 days post-infection with *P. yoelii* 17XNL, although we did not find significant difference between metformin-treated and untreated mice (**Figure S5B**).

Metabolic Changes and Signaling in γδ T Cells in Metformin-Treated Mice

Since metformin can modulate cellular metabolism through a mild, but specific inhibition of the respiratory chain complex 1 (20), we examined the metabolic function of γδ T cells in metformin-treated mice. Glucose uptake was monitored using 2NBDG, and mitochondrial mass was monitored with Mito FM staining (**Figure 7** and **Figure S1**). The γδ T cells and αβ T cells were each divided into three subgroups of cells using 2NBDG^{hi} Mito^{hi}, 2NBDG^{lo} Mito^{hi}, and 2NBDG^{lo} Mito^{lo} staining as markers (**Figures 7A,B**). The 2NBDG^{hi} Mito^{hi} T cells were primarily naïve T cells with a CD62L^{hi} CD44^{lo} phenotype and were the primary γδ T cells in uninfected mice. The 2NBDG^{lo} Mito^{hi} cells consisted of both naïve and activated T-cells, whereas the 2NBDG^{lo} Mito^{lo} cells were a minor population that was mainly a CD62L^{lo}-activated population (**Figure S6**). In the infected mice, the proportion of 2NBDG^{hi} Mito^{hi} cells decreased and the 2NBDG^{lo} Mito^{hi} cells became the major population of γδ T cells. The levels of 2NBDG and MitoFM in the 2NBDG^{lo} Mito^{hi} γδ T cells of the infected mice were higher compared to those in naïve mice, suggesting that glucose uptake and mitochondrial mass increased in activated γδ T cells of the infected mice (**Figures 7A,C**). However, we did not observe any significant differences in 2NBDG or MitoFM levels

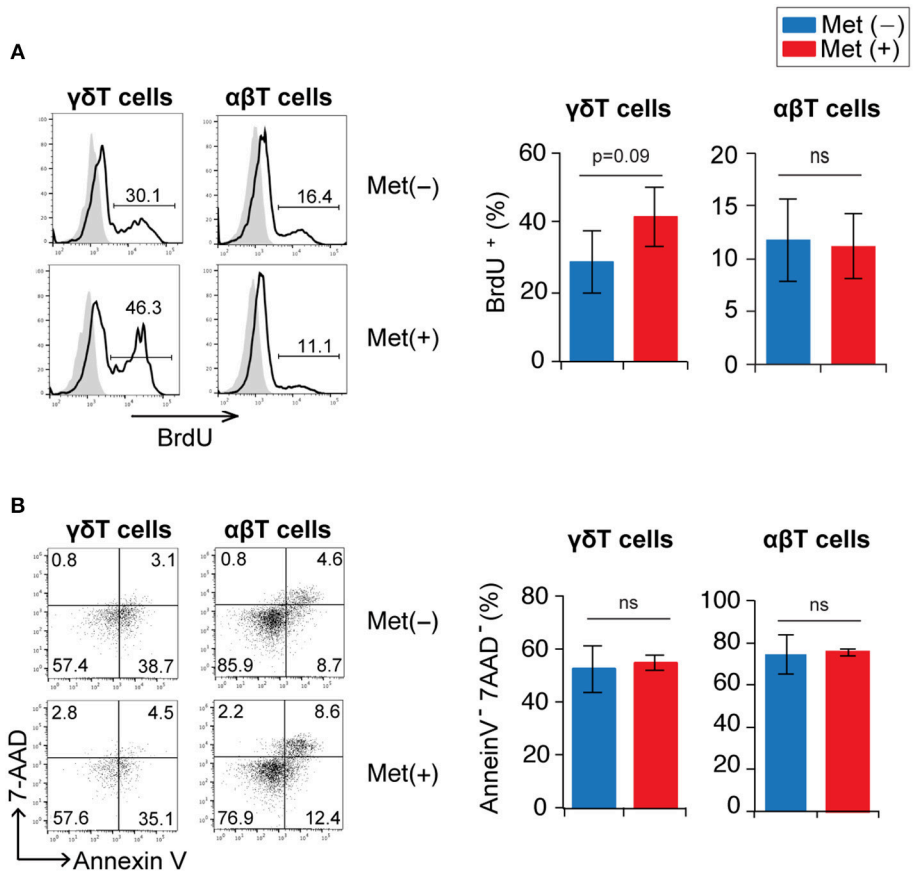

FIGURE 4 | Proliferation and survival of γδ T cells in *Plasmodium yoelii* 17XNL-infected mice by metformin. B6 mice were infected with *P. yoelii* 17XNL and treated with metformin (Met^+) or not (Met^-), as indicated. One day prior to analysis, mice received an intraperitoneal injection of BrdU. Mice were sacrificed at 16 or 18 d post-infection (3 mice/group). **(A)** Spleen cells were surface stained with anti-CD3 and anti-TCRγδ mAbs and stained intracellularly with an anti-BrdU mAb. BrdU-staining profiles in γδ ($CD3^+TCR\gamma\delta^+$) and αβ ($CD3^+TCR\gamma\delta^-$) T cells are shown. The numbers shown in the flow cytometry histograms indicate the proportion of cells within the region of the line. The proportion of $BrdU^+$ cells in the experiments were pooled for the bar graphs ($n = 6$). **(B)** Spleen cells were stained with anti-CD3 and anti-TCRγδ mAbs, annexin V, and 7-AAD. Annexin V vs. 7-AAD staining profiles in γδ ($CD3^+TCR\gamma\delta^+$) and αβ ($CD3^+TCR\gamma\delta^-$) T cells are shown. The numbers indicate the proportions of cells in each quadrant. The proportions of annexin $V^-7\text{-}AAD^-$ cells in the experiments were pooled ($n = 6$). Statistical significance was assessed using the unpaired *t*-test with Welch's correction (ns; not significant).

between the γδ T cells from metformin-treated and untreated mice.

Since metformin is reported to activate AMPK and inhibit the mTOR pathway (20, 25), we next examined AMPK activity and the mTOR pathway in γδ T cells (**Figures 8A,B**, **Figure S1**). However, we did not detect differences in the levels of AMPK and mTOR phosphorylation between metformin-treated and untreated mice by flow cytometry. Increased levels of phospho-S6, which is a downstream molecule of the mTOR pathway, was observed in the metformin-treated mice compared with that in the untreated mice, but the difference was modest. Thus, the effects of metformin on AMPK-mTOR pathway in γδ T cells appeared to be mild.

Improved Protection in Metformin-Treated Mice Was Independent of γδT Cells

To examine whether the increased number of γδ T cells contributed to the improved clearance of parasites in metformin-treated mice, we depleted γδ T cells *in vivo* by treating the mice with an anti-TCRγδ mAb prior to infection with *P. yoelii* 17XNL (**Figure 9**). In mice treated with the anti-γδ TCR mAb, the population of γδ T cells was almost completely depleted (**Figure 9B**). The depletion of γδ T cells did not affect the levels of parasitemia in either the metformin-treated or untreated mice, and reduced parasitemia was observed irrespective of the presence or absence of γδ T cells. We therefore concluded that the improved clearance of parasites in the metformin-treated mice was independent of γδ T cells.

DISCUSSION

γδ T cells are unique cellular subsets of T cells that express rearranged TCR γδ and have distinct recognition properties, distributions, and functions from those of T cells expressing TCR αβ (44). Many of the γδ T cells are distributed in the front line of defense against infection, such as in skin epithelial

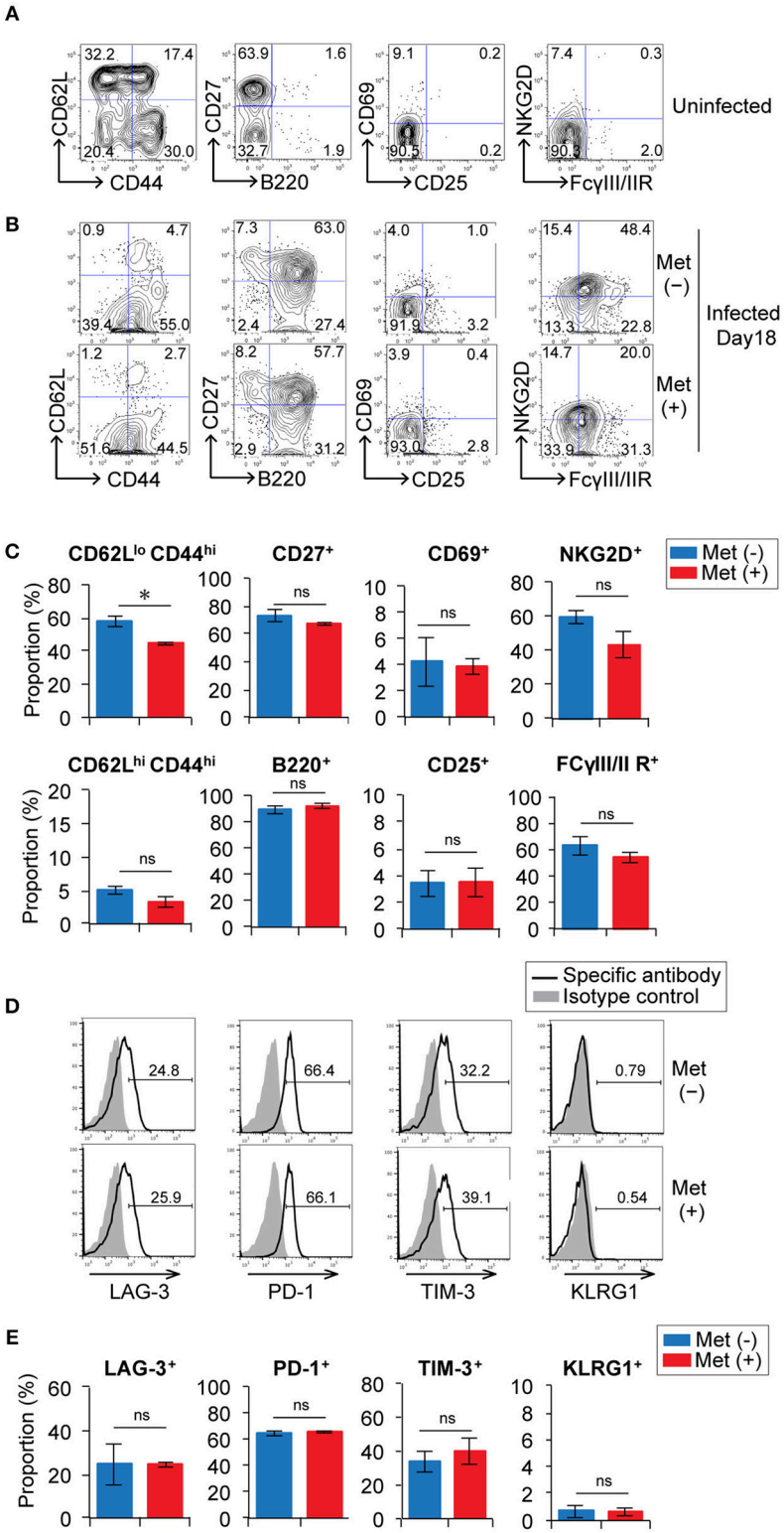

FIGURE 5 | Expression of activation markers and inhibitory receptors on γδ T cells in *Plasmodium yoelii* -infected mice treated with metformin. B6 mice were mock-infected **(A)** or infected with *P. yoelii* 17XNL and treated with metformin (Met^+) or not (Met^-), as indicated **(B)**. At 18 d post-infection, spleen cells were stained for CD3, TCRγδ, and other cell-surface markers **(A–C)** and inhibitory receptors **(D,E)**. Representative plots of these molecules on $CD3^+$ $TCR\gamma\delta^+$ cells **(B,D)** and the proportions of the positive γδ T cells are shown **(C,E)**. The data shown represent two independent experiments (3 mice/group) with similar results. Statistical significance was assessed using the unpaired *t*-test with Welch's correction (*$p < 0.05$; ns, not significant).

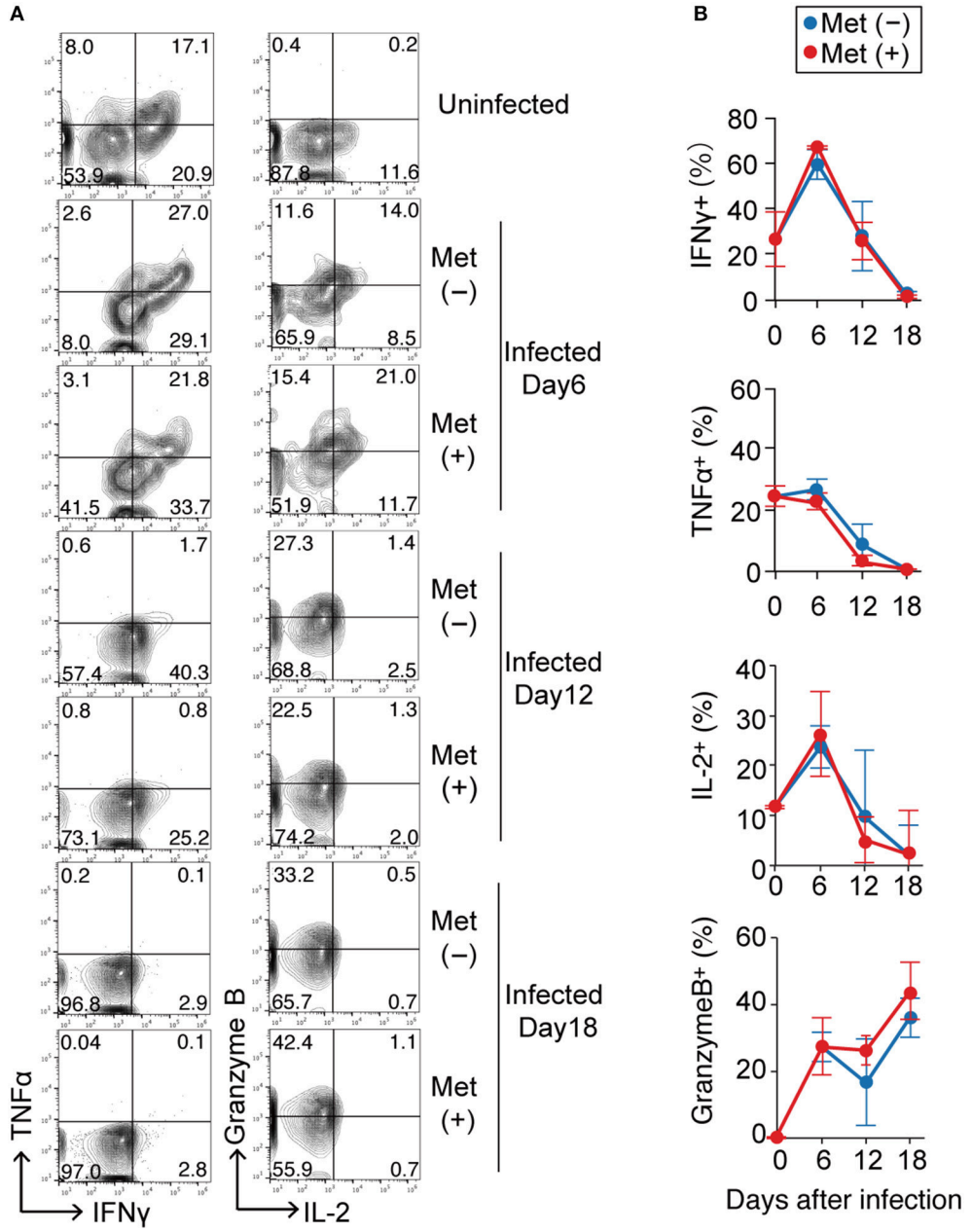

FIGURE 6 | Cytokine production by γδ T cells in *Plasmodium yoelii*-infected mice treated with metformin. B6 mice were infected with *P. yoelii* 17XNL and were treated with metformin (Met^+) or not (Met^-), as indicated. Splenocytes from uninfected and infected mice at 6, 12, or 18 d post-infection were stimulated with PMA and ionomycin for 4 h, surfaced stained for CD3 and TCRγδ, and intracellularly stained for IFNγ/TNFα or IL-2/Granzyme B. Representative plots of these molecules on $CD3^+$ $TCR\gamma\delta^+$ cells **(A)** and the proportions of cytokine-positive cells (%) in metformin-treated (red) and untreated (blue) mice **(B)** are shown. Each time point represents three mice that were analyzed. Statistical analysis was performed using the unpaired *t*-test with Welch's correction. No significant difference was observed between the metformin-treated and untreated mice.

tissue and the gastrointestinal tract, and they respond rapidly to changes in their environment and play critical roles in maintaining homeostasis in these tissues (45). Results from the current study demonstrated that γδ T cells expanded during the later phase of infection with *P. yoelii* 17XNL, which was consistent with previous studies (13–16). The expansion was strongly promoted by the administration of metformin, a drug commonly used for treating type-II diabetes. This increase in the number of γδ T cells occurred primarily in cells expressing Vγ1 or Vγ2, which localized to the red pulp of the spleen, and we suggest that it was likely mediated by increased proliferation rather than from reduced apoptosis. The selective expansion

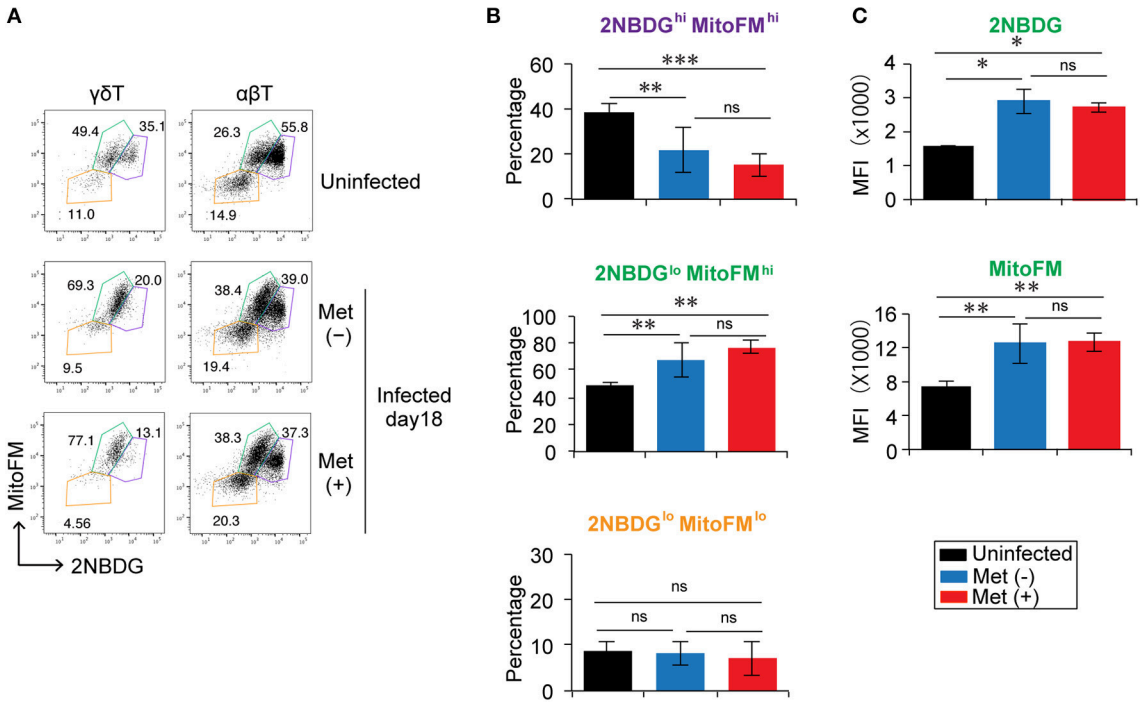

FIGURE 7 | Reduction in the glucose uptake by γδ T cells in *Plasmodium yoelii* 17XNL-infected mice. B6 mice were infected with *P. yoelii* 17XNL and treated with metformin (Met^+) or not (Met^-), as indicated. Spleen cells from uninfected and infected mice at 18 d post-infection were incubated with 2NBDG and then stained with an anti-CD3 and anti-γδTCR mAb in combination with MitoFM. The 2NBDG vs. MitoFM profiles of γδ T cells ($CD3^+$ $TCR\gamma\delta^+$) and αβ T cells ($CD3^+$ $TCR\gamma\delta^-$) **(A)**, the proportions of $2NBDG^{hi}$ $MitoFM^{hi}$ (purple), $2NBDG^{lo}$ $MitoFM^{hi}$ (green), and $2NBDG^{lo}$ $MitoFM^{lo}$ (orange) populations in $CD3^+TCR\gamma\delta^+$ cells **(B)**, and the mean fluorescence intensity (MFI) of $2NBDG^{lo}$ $MitoFM^{hi}$ cells **(C)** are shown (3 mice/group). The data shown represent two independent experiments (3 or 4 mice/group) with similar results. Statistical significance was assessed using one-way ANOVA with Tukey's *post-hoc* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, not significant).

of γδ T cells suggested that TCR signaling played a role in the expansion of these cells. The expansion of $V\gamma1^+$ γδ T cells in mice infected with the *P. berghei* XAT strain was reported previously (46) and suggests a recognition of malaria antigens by the Vγ1 TCR. The nature of the *Plasmodium* antigens recognized by γδ T cells was not determined. We think that it is likely that metformin enhanced proliferation of γδ T cells that were activated in response to *P. yoelii* antigens.

Metformin inhibits respiratory-chain complex 1 in mitochondria and, thus, modulates the metabolic function of cells in a primarily AMPK-dependent manner (20, 25, 47). However, we were unable to detect changes in the phosphorylation of AMPK or mTOR in γδ T cells from mice treated with metformin by flow cytometry, even though the effect of metformin on the activation of AMPK were previously confirmed (28). This result suggests that the effect of metformin on AMPK activation was modest at best under the experimental conditions used in our study. However, we did detect upregulated phosphorylation of S6, a downstream target of mTOR. The level of mTOR phosphorylation was modest when compared with that observed in $CD8^+$ αβT cells following TCR-mediated activation (48). Thus, we suspect that the mTOR pathway might be upregulated by metformin treatment, even though the increase in mTOR phosphorylation was undetectable by flow cytometry using our conditions. This scenario contradicts the general understanding of metformin activity on AMPK activation, which inhibits the mTOR pathway. An alternative possibility is that the upregulation of S6 phosphorylation occurred independently of mTOR activation. Further study is needed to determine the molecular signaling events that occur in γδ T cells leading to their expansion in *P. yoelii*-infected metformin-treated mice. In either case, our study showed that metformin-treatment resulted in an expansion of γδ T cells during the late phase of the infection with malaria parasites, while the number of αβT cells in the spleen remained low, suggesting that the effect of metformin on γδ T cells was distinct from that on αβ T cells.

It is known that γδ T cells secrete cytokines such as IFN-γ and TNF-α and produce granzymes, which help protect against pathogen invasion and also help maintain tissue homeostasis (42, 44, 49). Accordingly, γδ T cells upregulated the production of IFN-γ and IL-2 during the early phase of *P. yoelii* infection at a time when their numbers remained low. However, their ability to produce cytokines continued to decrease after 6 d post-infection and was almost lost by 18 d post-infection, at a point when the number of γδ T cells had clearly increased. In contrast, their ability to produce granzyme B continuously increased. These γδ T cells expressed the inhibitory receptors LAG-3, PD-1, and TIM-3, which implied that these cells entered

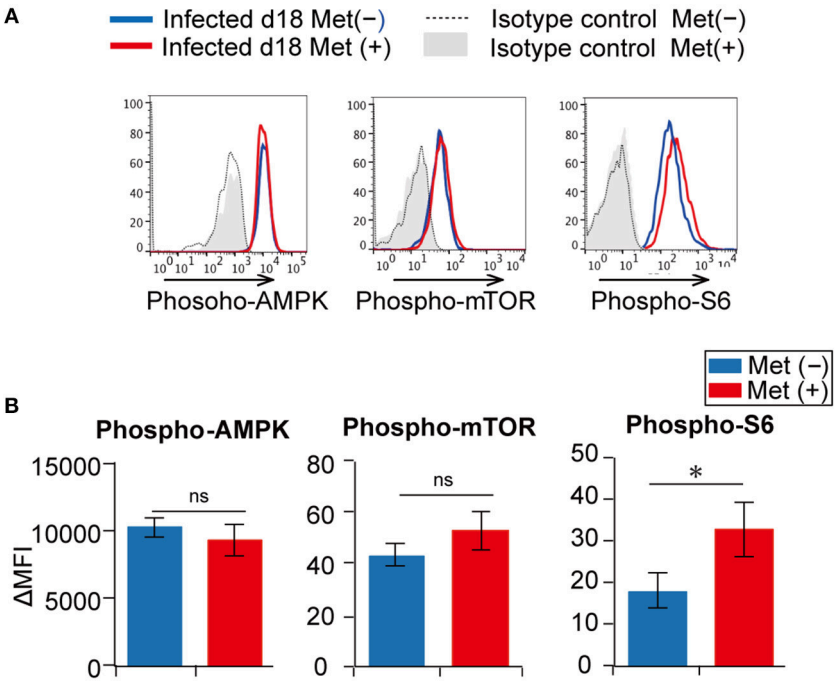

FIGURE 8 | Activation of the AMPK and mTOR pathways in γδ T cells from *Plasmodium yoelii* 17XNL-infected mice. B6 mice were infected with *P. yoelii* 17XNL and treated with metformin (Met^+) or not (Met^-), as indicated. Splenocytes from metformin-treated (red) and untreated (blue) mice were surface stained for CD3, TCRβ, and TCRγδ, and intracellularly stained for phospho-AMPK, phospho-mTOR or phospho-S6 **(A)**. Isotype controls for metformin-treated (gray shadow) and untreated mice (dotted line) are shown. Differences in mean fluorescent intensities (ΔMFIs) between experimental samples and isotype controls in metformin-treated (red) and untreated (blue) mice are summarized **(B)**. The data shown represent two independent experiments (3 mice/group) with similar results. Statistical significance was assessed using the unpaired *t*-test with Welch's correction (*$p < 0.05$; ns, not significant).

an exhausted state as previously described (46). Metformin treatment did not affect the expression of these inhibitory receptors or the functions of the γδ T cells during infection. Taken together, these data suggest that γδ T cells increased in number during the late phase of *P. yoelii* infection but were functionally impaired due to being in an exhausted state. Therefore, these γδ T cells may not have been able to participate in protective immune responses against infection. In fact, the depletion of γδ T cells did not affect the levels of parasitemia in either the metformin-treated or untreated mice during the course of infection with *P. yoelii*.

Therefore, the question remains why treating mice with metformin reduced the levels of parasitemia. One possibility is that metformin directly inhibited the growth of *Plasmodium* parasites *in vivo*. Since metformin is a derivative of the biguanide drugs, whose anti-*Plasmodium* action was previously shown, we are unable to exclude this possibility. An alternative, but not mutually exclusive, possibility is that metformin acted on other immune system cells such as αβ T cells and B cells in the infected mice. In this study, we observed slight, but significant effects of metformin on the serum levels of anti-parasite IgG, the number of dendritic cells in the spleen on day 12 of the infection (**Figure S3**), and the proportion of $CD62L^{hi}$ $CD44^{hi}$ αβ T cells on day 18 of the infection (**Figure S4**). In addition, our preliminary data suggested that metformin treatment did not reduce parasitemia in Rag-2 gene-knockout mice, which lack an adaptive immune system (data not shown). Therefore, we think that the effect of metformin on reducing parasitemia depends on the host adaptive immune system, which enhances protective immunity. Further study is required to determine the mechanisms underlying the reduction of parasitemia in metformin-treated mice during *P. yoelii* infection.

In humans, γδ T cells recognize *Plasmodium* antigens and expand in a manner dependent on $CD4^+$ T cells or cytokines, including IL-2, IL-4, and IL-15 (50). These γδ T cells are cytolytic to *Plasmodium* parasites and produce cytokines, and could play both protective and pathogenic roles in patients infected with *P. falciparum* (8, 9). Since metformin is used as an anti-diabetic drug in malaria-endemic regions, this drug may enhance the increase of γδ T cells in patients infected with *Plasmodium* parasites. Since these γδ T cells are functional, they might affect the clinical outcome of the infection in either positive or negative manner. Therefore, the findings of this study suggest the importance of monitoring malaria disease progression in endemic regions when patients with diabetes are treated with metformin.

The γδ T cells represent a unique T cell subset whose antigen recognition and functions are not clearly understood. These cells expand during *Plasmodium* infection and play critical protective

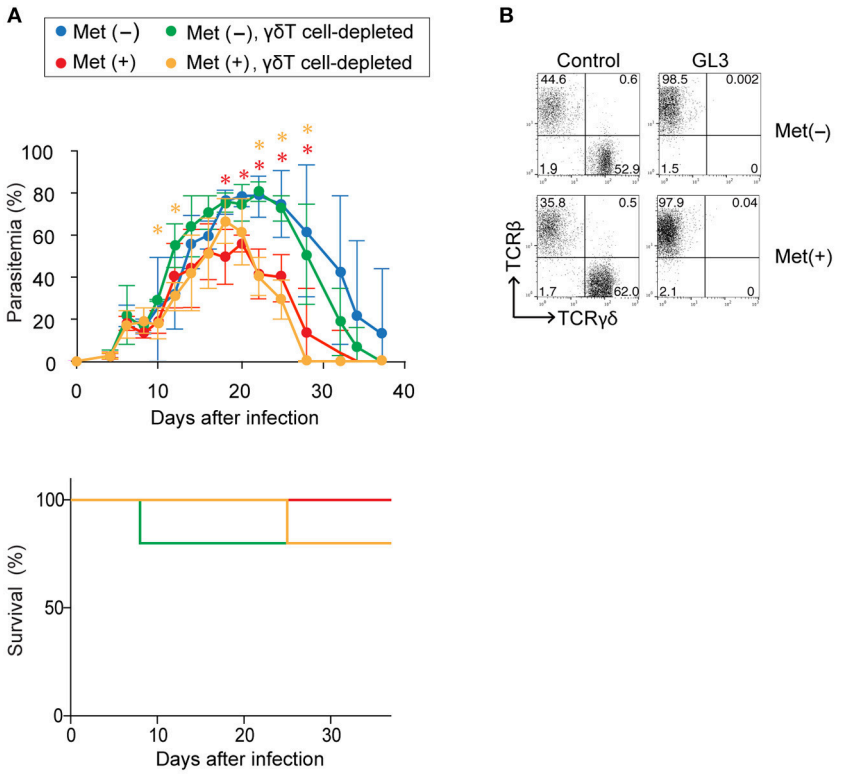

FIGURE 9 | The role of γδ T cells in enhanced protection against *Plasmodium yoelii* 17XNL by metformin. B6 mice were infected with *P. yoelii* 17XNL and treated with metformin (Met^+; red, orange) or not treated (Met^-; blue, green). The mice were injected with an anti-γδTCR mAb (500 μg; orange, green) or PBS (red, blue) on day−1, 0, or 1 relative to infection and twice a week following infection. **(A)** Parasitemia and survival was monitored every few days. The data shown represent two independent experiments (4–5 mice/group) with similar results. Statistical significance in the differences between the metformin-treated and untreated mice among γδ T cell-sufficient (red asterisk) and γδ T cell-depleted mice (orange asterisk) was assessed by the unpaired *t*-test with Welch's correction (*$p < 0.05$). **(B)** At 40 d post-infection, splenocytes of mice treated (GL3) or not treated (control) with an anti-γδTCR mAb were stained for CD3, TCRγδ, and TCRβ expression to confirm the depletion. Representative plots of the $CD3^+$-gated population are shown.

functions by regulating both innate and adaptive immune responses (16, 49). We showed that metformin promoted the expansion of γδ T cells, but not αβ T cells, during the later phase of *Plasmodium* infection, highlighting the fact that the proliferation of γδ T cells may be regulated in a manner metabolically distinct from that of αβT cells. Further study on the metabolic function of γδ T cells and its effects on their function and clonal expansion may reveal novel strategies in enhancing protective immunity and promoting memory immune responses.

AUTHOR CONTRIBUTIONS

MM designed and performed the experiments, interpreted the results, and wrote the manuscript. GB performed the experiments. DK and MA provided technical advice. HU provided conceptual advice. KY designed the experiments, interpreted the results, wrote the manuscript, and supervised this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02942/full#supplementary-material

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REVIEW
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Comprehensive Review of Human *Plasmodium falciparum*-Specific CD8+ T Cell Epitopes

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Control of malaria is an important global health issue and there is still an urgent need for the development of an effective prophylactic vaccine. Multiple studies have provided strong evidence that *Plasmodium falciparum*-specific MHC class I-restricted CD8+ T cells are important for sterile protection against *Plasmodium falciparum* infection. Here, we present an interactive epitope map of all *P. falciparum*-specific CD8+ T cell epitopes published to date, based on a comprehensive data base (IEDB), and literature search. The majority of the described *P. falciparum*-specific CD8+ T cells were directed against the antigens CSP, TRAP, AMA1, and LSA1. Notably, most of the epitopes were discovered in vaccine trials conducted with malaria-naïve volunteers. Only few immunological studies of *P. falciparum-specific* CD8+ T cell epitopes detected in patients suffering from acute malaria or in people living in malaria endemic areas have been published. Further detailed immunological mappings of *P. falciparum*-specific epitopes of a broader range of *P. falciparum* proteins in different settings and with different disease status are needed to gain a more comprehensive understanding of the role of CD8+ T cell responses for protection, and to better guide vaccine design and to study their efficacy.

Keywords: malaria, *Plasmodium falciparum*, CD8+, T cell epitope, HLA, restriction, cytotoxic T cells

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INTRODUCTION

Malaria remains one of the pressing global health issues with ∼216 million cases per year (1). The most virulent *Plasmodium* species, *Plasmodium falciparum (P. falciparum)*, accounts for the vast majority of the 445,000 deaths which occurred in 2016 due to malaria. Rising rates of drug (parasite-related) and insecticide (vector-related) resistance underscore the urgent need for an effective vaccine (2). The feasibility of achieving sterile protection by vaccination has already been demonstrated more than 50 years ago by experiments that could induce sterile immunity using irradiated sporozoites in mice (3) and in humans (4, 5). But the immunization of large numbers of individuals using sporozoites remains challenging and there is limited evidence for durable, high-level efficacy against natural exposure (6, 7). So far, the development of a subunit vaccine (that contains only the antigenic parts of a pathogen) has not succeeded despite decades of research (8, 9). In order to achieve sterile protection against *P. falciparum* infection, a better

understanding of host-parasite interaction, and correlation of a protective *P. falciparum*-specific immune response is necessary. In particular, detailed knowledge of the *P. falciparum*-specific CD8+ T cell epitope repertoire is needed to further optimize vaccine design and to improve immune monitoring of future clinical malaria vaccine trials. In this review, we summarize and discuss all of the *P. falciparum*-specific CD8+ T cell epitopes in humans that have been identified so far, their potential significance as well as potential knowledge gaps.

THE RTS,S VACCINE AND CSP-SPECIFIC CD8+ T CELLS

There are currently several vaccine trials under way. However, recently published trials could only demonstrate suboptimal effectivity (10, 11). A malaria subunit vaccine, called the RTS,S vaccine (Mosquirix) targeting the CSP protein is the most advanced vaccine (12). Unfortunately vaccine efficacy was only modest (13), waned over time, and was not detectable any more three years after vaccination (11). Elevated IFNγ-levels after antigen-specific short-term cultivation and re-stimulation were detected by ELISPOT in subjects vaccinated with the RTS,S and CSP constructs and their levels decreased with waning protection (14). These studies demonstrate the importance of *P. falciparum*-specific T cells and the need to improve future strategies to induce a strong, broad and long-lasting vaccine-induced *P. falciparum*-specific CD8+ T cell response (15).

CHALLENGES OF MALARIA-VACCINE DEVELOPMENT

Some of the particular obstacles of the development of a protective malaria vaccine are:

a) The large size of the 23-megabase nuclear genome which consists of 14 chromosomes encoding for about 5,300 genes (16),
b) the great genetic heterogeneity of some regions of those genes, and
c) the complex life cycle and expression pattern of these genes (17),

rendering malaria vaccine design more complicated than for most viral and bacterial infections.

A detailed understanding of the life cycle of the parasites and determination of the immune responses that confer protection after natural infection will provide important insights for future vaccine development. Within the human host, two main malaria life cycle stages are completed (18): the pre-erythrocytic stage and the blood stage. The pre-erythrocytic stage can be divided into an early sporozoite stage and the liver stage. During the early sporozoite stage, sporozoites are injected into the skin, enter the blood stream, and consecutively infect hepatocytes. This stage lasts minutes to hours (19). The ensuing liver stage consists of asexual replication and the maturation of sporozoites into schizonts within the hepatocytes, which lasts for ∼1 week in humans in *P. falciparum* malaria (20). The subsequent blood stage is initiated with the release of merozoites that infect red blood cells (RBCs) and is the period in which clinical symptoms occur. These symptoms are partly induced by excessive host immune responses [reviewed in Artavanis-Tsakonas et al. (21)]. A small number of parasites in the blood develop into sexual-stage gametocytes, which can be taken up by the mosquito and continue the cycle of infection. The proteins that are expressed by plasmodia are life cycle stage specific: During the liver stage different (surface-) proteins like the circumsporozoite protein (CSP), liver stage antigens (LSA) and thrombospondin-related anonymous protein (TRAP) are expressed, while the merozoite surface protein (MSP) are expressed during the blood stage. The apical membrane antigen 1 (AMA1) is present in both stages (22). *P. falciparum*-specific T cells are primed within different immunological environments which might influence breadth and quality of the *P. falciparum*-specific response against the different antigens. It has been proposed that parasite-specific CD8+ T cells are mostly primed during the liver stage, while *P. falciparum*-specific CD4+ T cells enable clearance of the blood stage infection (23). CD8+ T cells are probably not important effector cells during the blood stage because the parasite-infected human erythrocytes do not display MHC class I molecules (24).

Nevertheless, naturally acquired immunity against malaria is primarily directed against blood stage antigens (mainly mediated via antibodies) and therefore prevents clinical disease but not the infection of hepatocytes (6, 7, 25), suggesting that there is no, or only little naturally acquired immunity against the pre-erythrocytic stage (26, 27). Of note, the pre-erythrocytic stage represents a bottleneck for the parasite (and is an ideal target for the host specific immune response) because of the relatively low number of parasites passing through this phase (∼100 sporozoites, only) (28). A strong, specific-CD8+ T cell response directed against the liver stage would optimally prevent the parasite's transition to the blood stage and clinical symptoms and disease could be avoided (15).

THE *P. FALCIPARUM*-SPECIFIC CD8+ T CELL RESPONSE

Plasmodia, like other pathogens, induce a variety of immune effector responses: *P. falciparum*-specific antibodies, parasite-specific CD8+, and CD4+ T cells, as well as certain cytokines have been implicated as important effectors [reviewed in Stevenson and Riley (29) and Dobaño and Moncunill (30)]. The current review will focus on human *P. falciparum*-specific CD8+

Abbreviations: AA, amino acids; AMA1, apical membrane antigen; APC, antigen presenting cells; CSP, circumsporozoite protein; CTL, cytotoxic T lymphocytes; ELISA, enzyme linked immunosorbent assay; EXP1, exported protein one; GLURP, glutamate-rich protein; HSPGs, heparan sulfate proteoglycans; HLA, human leukocyte antigens; ICS, intracellular staining; IEDB, The Immune Epitope Database and Analysis Resource; IFNγ, interferon gamma; Il, interleukin; LSA, liver stage antigen; MHC, major histocompatibility complex; MSP, merozoite surface protein; P, Plasmodium; Pf, Plasmodium falciparum; PfEMP, erythrocyte membrane protein; Pfs16, sexual stage specific protein; RF, response frequency; STARP, sporozoite threonine and asparagine-rich protein; TNFα, tumornecrosis factor alpha; TRAP, thrombospondin-related antigen.

T cells since there is strong experimental evidence in rodent and primate models as well as in humans that CD8+ T cells play a major role in providing protection:

- Sterile immunity using irradiated sporozoites in mice could be induced. This immunity was specific against the liver phase and later inoculation with parasitized red blood cells led to infection (3).
- Mice and rhesus monkeys were protected by immunization with attenuated sporozoites. This immunity was abrogated by experimental depletion of CD8+ T cells (31–34).
- The transfer of CSP-specific CD8+ T cell clones, as well as the transfer of a defined CD8+ T cell epitope conferred protection to malaria-naïve mice (35–39). Likewise, TRAP-peptides from *P. berghei* and a novel liver stage antigen MIF-4-like protein peptide Kb-17 have been able to elicit a CD8+ T cell-dependent response against murine malaria (40, 41).
- Immunity provided by antibodies was shown to be suboptimal most likely because blood-stage surface antigens show great variability (42, 43). Mouse models demonstrated that a robust CD4+ and CD8+ T cell response improves immunity since these T cell responses target internal antigens that are more likely to be conserved (44–46).
- Mouse models could show that induction of extremely high numbers of memory CD8+ T cells were a prerequisite for solid, sterile protection (47, 48).
- CD8+ T cells have shown to be important effectors that form clusters around infected hepatocytes and destroy them (49, 50). The importance of liver resident CD8+ T cells in protective immunity induced by attenuated *P. berghei* sporozoites has also been reported (51, 52). Mounting evidence suggests that effector CD8+ T cells eliminate the parasites without direct contact with infected hepatocytes via cytokine release (53). This model is also supported by the lymphogenic features of the liver (53).
- In humans, the inoculation with intact sporozoites (that were not attenuated) led to an increase of parasite-specific pluripotent effector memory T cells (54). The levels of *P. falciparum*-specific CD8+ T cells were also higher in those subjects who were protected after immunization with irradiated sporozoites compared to unprotected subjects (55).
- The polymorphism of CSP is primarily located in the region of identified CTL and T helper epitopes (56).
- An association with HLA class I and the course of the disease has been described showing that HLA-B*53, was associated with resistance to severe malaria (57). MHC class I-dependent presentation of antigens in *P. berghei* malaria was also demonstrated in the mouse model (58).
- Humans immunized with irradiated sporozoites or naturally exposed to malaria can generate a CTL response to pre-erythrocytic-stage antigens (8, 59–63).
- In mouse models, an excessively strong CD8+ T cell response has been associated with the development of cerebral malaria (64), and a deeper understanding of the CD8+ T cell repertoire may have implications beyond vaccine development and could be relevant for the clinical course of this disease in humans.

The mechanism of protection by CD8+ T cells is thought to be partly cytokine-mediated by interferon-γ (IFNγ) (31) and tumor necrosis factor (TNF) [reviewed in Dobaño and Moncunill (30)] that both inhibit parasite development. Perforin and granzymes kill infected hepatocytes through direct lysis (39), (65–68). IFNγ responses directed against pre-erythrocytic stage antigens of *P. falciparum* are associated with higher hemoglobin levels and significantly reduced prevalence of severe anemia (69) and are associated with significant resistance to re-infection (70, 71). It could also be shown that age-related cumulative exposure to *P. falciparum* increases the frequency of IFNγ responses (72, 73). This demonstrates the importance of IFNγ production for protection against malaria.

CHALLENGES OF INVESTIGATING THE *P. FALCIPARUM*-SPECIFIC CD8+ T CELL RESPONSE

So far, the breadth and specificity of the parasite-specific CD8+ T cell response in malaria has only been poorly described. One problem of the mapping of *P. falciparum*-specific T cell responses is the comparatively low *ex vivo* frequency of circulating peripheral *P. falciparum*-specific T cells (74, 75). There are several proposed hypotheses to explain low frequencies and responses in peripheral blood samples:

- Lymphopenia occurs during acute malaria infection [reviewed in Scholzen and Sauerwein (76)]. T lymphocytes are thought to migrate into different tissues, e.g., the liver (8, 77).
- There is a high genetic diversity between different *P. falciparum* strains with high mutation rates of several antigenic regions of the genome that complicates detection of T cells (**Supplementary Figures 1–4**). Additionally, no optimized consensus sequence for screening purposes of T cell responses against malaria proteins has been designed as of now (78–80). This extreme genetic diversity, particularly of the surface antigens, makes the detection of *P. falciparum*-specific T cells difficult (78–80).
- The immunoregulatory effects of a blood stage infection are thought to influence the priming of an adaptive immunity against the pre-erythrocytic stage (81–83).
- *P. falciparum* contains thousands of antigens that could serve as potential T cell epitopes. This sheer breadth of epitopes primed during an acute infection could also explain the low frequencies of specific T cells against an individual T cell epitope (84).
- The inability to generate new responses against emerging variants (original antigenic sin), is a phenomenon that has been described for several pathogens like influenza, HIV, and the hepatitis B virus (85–91). Malaria can be caused by infection with multiple *Plasmodium* strains (92, 93) and limited responsiveness of CD8+ T cells in coinfection with different *Plasmodium* strains could be shown (94) similar to the limited cross-genotype responsiveness after infection with different HCV genotypes (95).

- The human immune T cell response is modulated and dampened by the parasite through several mechanisms (83, 96). Walther et al. showed that regulatory T cells were rapidly induced following blood-stage infection and were associated with a burst of TGF-β production, decreased proinflammatory cytokine production, and decreased antigen-specific immune responses (96). Mackroth et al. demonstrated that acute *P. falciparum* malaria induced *P. falciparum*-specific PD1+CTLA4+CD4+ T effector cells that co-produced IFNγ and IL-10 and inhibited other CD4+ T cells (83). In rodent malaria it could be shown that PD-1 mediates up to 95% reduction in numbers and functional capacity of parasite-specific CD8+ T cells (97).
- Evasion mechanisms like Kupffer cell apoptosis and reduced expression of major histocompatibility complex (MHC)-I also resulted in T cell tolerance (98). Additionally, a reduced APC (antigen presenting cell) function of the Kupffer cells from sporozoite-infected mice was shown which might also explain the comparatively low magnitude of *P. falciparum*-specific T cell responses detected in malaria patients (99).

THE ROLE OF HLA ALLELES IN MALARIA

Certain human leukocyte antigens (HLAs) like HLA-B53, DRB*13:02, and DQB*05:01 are associated with protection from severe malaria (84, 100). This suggests that selection of particular epitopes might be associated with better control of parasitaemia. Gabonese children carrying the HLA class II allele DQB1*0501 had a higher frequency of IFNγ-responses to LSA1 T cell epitopes, compared with non-carriers, and were better protected against malaria anemia and re-infections (100).

APFISAVAA (LSA3), **EPKDEIVEV** (LSA3), and **KPIVQYDNF** (LSA1) are the only known CD8+ T cell epitopes with HLA-B53 restriction and are located on the liver stage antigen 1 (LSA1) and the liver stage antigen 3 (LSA3) (23, 63, 100–103).

The genes coding the HLA molecules are the most polymorphic genes in humans and several observations indicate that this is due to selective immunological pressure (84). This polymorphism leads to varying degrees of protection against infectious pathogens (but also to association between MHC types and autoimmune diseases) (104). *P. falciparum* infection has been a major selective force in human evolution, especially in West Africa, where the HLA alleles HLA-A*53, DRB1*13:02, and DQB1*05:01 are more frequent than in the rest of the world (allelefrequencies.net) (105). But *P. falciparum* contains thousands of different potential T cell epitopes and multiple immune responses are evoked by a single infection (74). This implies that most of these responses are possibly of limited protective value and only a few epitopes will elicit strong, long lasting and protective immune answers (84). However, longitudinal studies about the relationship between HLA alleles, cytokine patterns, and the outcome of *P. falciparum* infection are rare (100) and other protective HLA alleles have not yet been identified in humans.

METHODS FOR DETECTION OF ANTIGEN-SPECIFIC T CELLS

Over the last 20 years, the methodologies of T cell detection have undergone considerable changes. *P. falciparum*-specific T cells have been detected by a number of different assays that differ by practicability, price, sensitivity, and functional read-out. Different assays have different advantages and disadvantages, which has to be taken into account when comparing results of different studies that have characterized *P. falciparum*-specific T cell responses over the last decades. The most commonly used techniques for T cell analysis in malaria are shown in **Table 1**.

Potential difficulties of the generally low frequencies of the *P. falciparum*-specific T cells can be overcome by *in vitro* expansion and short-term culture methods. Numerous studies have cultivated PBMCs with the particular antigen for a few days up to 2 weeks to allow expansion of the specific T cells (74, 121). However, *in vitro* expansion alters the T cell function and phenotype to some extent. It is also difficult to extrapolate the original specific *ex vivo* T cell frequency after culture. An alternative approach to increase low frequencies of antigen-specific T cells is the quantitative pre-enrichment of target cells via magnetic cell separation (118). This approach allows phenotypic assessment of the specific T cells but has not yet been used in the context of malaria CD8+ T cell research.

RESOURCES FOR *P. FALCIPARUM*-SPECIFIC EPITOPE RESEARCH AND *IN SILICO* ANALYSIS

To date, there are several online *P. falciparum*-specific resources available on the internet that provide immunological information and/or data related to plasmodial antigens, or that offered links to clinical trial data. This section provides a brief overview of these important resources.

THE IEDB: AN ONLINE EPITOPE RESOURCE

The Immune Epitope Database and Analysis Resource (IEDB.org) was created by the National Institute of Allergy and Infectious Disease (NIAID) to provide the scientific community with a repository of freely accessible immune epitope data (122, 123). The IEDB contains data captured from the published literature, as well as data submitted through NIAID's high-throughput epitope discovery efforts. The IEDB includes antibody and T cell data from human, non-human primate, and rodent hosts, as well as other animal species, and encompasses epitopes associated with all infectious diseases, allergy, autoimmunity and transplantation and/or alloreactivity. In addition to antibody and T cell response data, the IEDB also contains MHC binding and MHC ligand elution data (mass spectrometry).

It thus provides a unique resource to inventory and analyze immunological data for a given pathogen or

TABLE 1 | Different methods for detection of *P. falciparum*-specific CD8+ T cells.

Method	Relevance for T cell detection in malaria	Advantages and limitations
Chromium-51 (51Cr) release assay	• Used for CD8+ T cell detection in malaria from 1991 to 2004 and the majority of published malaria-specific T cell epitopes were discovered by this method	• Specific for cytolysis • Sensitive (1 cell out of 100) • Specific infrastructure required (107) • Expensive, work, and time intensive
ELISA	• Few studies have used ELISA for detection of malaria-specific CD8+ T cell epitopes to date (70, 74, 100, 108)	• 400-fold less sensitive than ELISPOT • Analyzes cytokines and not direct cytolysis (109)
[3H]-thymidine incorporation assay	• Commonly used in cell proliferation assays but mostly for CD4+ T cell detection (110) • Only one study detected a CD8+ T cell epitope shorter than 20 AA (69)	• Error prone, unspecific at times • No info on *ex vivo* phenotype of T cell • Specific infrastructure required (111)
ELISPOT	• Sensitive and robust immunological method for enumerating antigen-specific lymphocytes (112, 113) • Not used for specific T cell detection in the field of malaria until 1999 (114)	• Sensitive, cost effective technology, easy to set up in tropical regions • CD4+ T cell depletion is necessary to link cytokine production to CD8+ T cells (115) • No information on *ex vivo* T cell phenotype
ICS	• Only two studies have used ICS (intracellular cytokine staining) technology for Plasmodium-specific CD8+ T cell mapping (116, 117)	• Cell, cost, and work intensive assay (118)
MHC class I-multimer	• Only two studies have performed MHC multimer staining of vaccine-induced *Plasmodium*-specific CD8+ T cell responses (119, 120)	• Sensitive and specific technology • Detection is uncoupled from function and thus T cells can be detected that do not produce cytokines at the time of examination (118) • HLA molecules of patients need to be determined • Expensive and error prone technology, few *P. falciparum*-specific MHC multimers have been established so far

disease and has been utilized to produce several meta analyses including a 2009 analysis of epitopes derived from *Plasmodium* (110).

Currently, the IEDB contains 3,000 unique epitopes derived from the genus *Plasmodium*. Of these data, species causing malaria, *P. falciparum*, *P. vivax,* and *P. malariae*, represent 88% of the total, with *P. falciparum*-specific determinants most prominent among them (2,332). *P. falciparum*-specific data include epitopes defined for more than 160 antigens, including both protein and non-protein (cell surface glycolipids) targets. Data related to human disease are most numerous, with 1,485 epitopes defined from 126 different plasmodial antigens and reported from 295 references. These were data derived from more than 3,900 immunoassays (2,205 T cell and 1,693 antibody). Data generated using murine models of disease (710 epitopes) and non-human primates (104 epitopes) are also available. At the level of immune response phenotype, there are currently 774 human antibody epitopes from 120 antigens and 843 T cell epitopes from 32 antigens, including linear and non-linear antibody epitopes, and class I (147) and class II (596). Of the 147 MHC class I/ CD8+ T cell epitopes, we included 132 epitopes in this review. Fifteen epitopes were excluded because they were longer than 20 amino acids (AA).

In addition to its epitope data repository, the IEDB hosts an array of epitope prediction and analysis tools, including those for T cell class I and II prediction (tools.iedb.org/main), such as TepiTool (124), the T cell class I pMHC immunogenicity predictor (125), and the CD4+ T cell immunogenicity prediction tool (126). Useful among the other analysis resources are the Immunome Browser, which maps epitope prominence along the antigen, the Epitope Cluster Analysis Tool 2.0, and the Epitope Conservation Analysis tool (127).

OTHER *P. FALCIPARUM*-SPECIFIC ONLINE RESOURCES

Informatic sites containing plasmodial antigen sequence and/or structural data include the *Plasmodium* Genomics Resource (plasmodb.org/plasmo) (128) and the *Plasmodium* vivax Structural Databank (PvaxDB) (scfbio-iitd.res.in/PvaxDB) (129). The Rodent Malaria genetically modified Parasites (RMgmDB) (pberghei.eu/index.php) site (130) contains genetically modified rodent malaria parasites that have been generated by many labs worldwide. The Malaria Data site (ebi.ac.uk/chembl/malaria) provides a searchable and downloadable public resource for targets, compounds, assays, and other data related to malaria research (131). Similarly, the Malaria Immunology database (malarimdb.org/) provides users an overview of the roles of the various immunological, metabolic vascular, and erythrocytic factors involved in blood-stage malaria and includes human patient data, as well as data from murine models of disease (132). The Malaria Atlas Project (map.ox.ac.uk) enables users to download, visualize and manipulate global parasite rate survey data (133). MalariaGen (malariagen.net) is a genomic epidemiology network for next generation DNA sequencing tools and technologies (134).

COMPREHENSIVE REVIEW OF *P. FALCIPARUM*-SPECIFIC CD8+ T CELL RESPONSES

While a great number of *P. falciparum*-specific T cell responses can be searched in the IEDB (122), a comprehensive review providing an overview of discovered CD8+ T cell epitopes in *P. falciparum* and discussing them in context is lacking, to date. Therefore, we conducted an exhaustive database (www.iedb.org) (Terms: *P. falciparum*; MHC Class I; Humans) and literature review (PubMed)[1] (Terms: *P.falciparum*; CD8+ T cells; epitopes; cytotoxic T cells; MHC class I) and summarized our findings in form of an epitope map (**Figures 1**–**7**). We defined an epitope as a *P. falciparum*-specific peptide of 20 AA (amino acids) or less that elicited a CD8+ T cell response. Fifteen epitopes that had shown to be restricted to MHC class I, but were longer than 20 AA, were excluded from our review (106, 116). Epitopes described for other *Plasmodium* species or T cell responses primed and detected in murine malaria infection were similarly not included in the current overview. Likewise, *P. falciparum*-specific CD4+ T cell responses were also not listed. The relative pattern of immune dominance or relative breadth and quality (e.g., cytokine pattern) of the response measured against each of the epitopes was also not assessed.

From the 147 CD8+ T cell epitopes found in the IEDB, we included 132. These epitopes were described in 36 different publications. Additional information about the studies, like the methods that were used, the experimentally assessed HLA restriction (if available) and the study population that was tested, was included for each epitope in **Supplementary Table 1**. We distinguished between four different types of study populations that were tested for specific CD8+ T cells:

1. Malaria-naïve volunteers immunized with *P. falciparum* antigens in a vaccine trial
2. Malaria-naïve volunteers immunized with attenuated sporozoites
3. Volunteers from a malaria endemic area naturally exposed to *P. falciparum*
4. Volunteers with known *P. falciparum* infection (acute or post clinical malaria).

The epitopes are then discussed in the context of the protein structure and their role in pathogenesis, as well as in terms of overall reported immunodominance. For this, response frequency (RF) data was used. RF represents the number of respondents over the number of subjects tested, when provided by the investigator for the respective assay. It has to be taken into account that some studies only included volunteers that carry the MHC allele HLA-A*02 (141). In this case the RF is not representative for a MHC diverse population.

The assessment of the breadth and specificity of a *P. falciparum*-specific immune response will be a useful tool for the field and provide better knowledge of the *P. falciparum*-specific T cell response on an epitope level. This will likely be crucial for the understanding of the exact role of CD8+ T cells for control of disease, for vaccine design and for monitoring of the CD8+ T cell response in future vaccine trials (142). Of the more than 5,000 *P. falciparum* proteins, *P. falciparum*-specific CD8+ T cell epitopes have only been described thus far for the following nine antigens:

1. Circumsporozoite protein (CSP)
2. Thrombospondin-related anonymous protein (TRAP)
3. Apical membrane antigen 1 (AMA1)
4. Liver stage antigen 1 (LSA1)
5. Liver stage antigen 3 (LSA3)
6. Circumsporozoite-related antigen (EXP1)
7. Merozoite surface protein 1 (MSP1)
8. Sexual stage specific protein 16 (Pfs16)
9. Sporozoite threonine and asparagine-rich protein (STARP).

The majority of published MHC class I restricted CD8+ T cell epitopes was detected on CSP, with 55 known epitopes. This corresponds to 42% of all CD8+ T cell epitopes. This is followed by TRAP with 28 CD8+ T cell epitopes (21%). Nineteen epitopes on AMA1, 13 epitopes on LSA1, 7 epitopes on LSA3, and 6 epitopes on EXP1 that are specific for MHC class I/ CD8+ T cells had been detected. For the antigens MSP1, Pfs16, and STARP only one CD8+ T cell epitope, respectively, has been found (63, 69, 74, 143).

The average response frequency of all assays combined (when provided) was 23% [2.2–100% (74)] (total number of assays: 228). An average of 2.4 [1–11 (144)] subjects responded and the average size of a study population was 10.3 subjects [1–45 (74)]. Looking only at the studies that included malaria-naïve volunteers (total number of assays: 97), an average of 26.8% [5.3–100% (145)] responded in an average group of 8.2 [1–19 (145)] subjects. The study cohorts with volunteers from a malaria endemic area had an average response frequency of 16.0% [2.2–100% (74)] (total number of assays: 91). The average size of these cohorts was 15.4 study subjects [1–45 (74)]. The study group of volunteers immunized with attenuated sporozoites was a lot smaller with an average of 3 [1–4 (146)] subjects tested. The average RF was 72.6% [50–100% (147)] (total number of assays: 40).

In the following section MHC class I-restricted, *P. falciparum*-specific CD8+ T cell epitopes that were published at least in three different publications and/or showed a response frequency of 40% or greater or other important features will be listed according to each of these proteins. **Supplementary Table 1**, which gives a more comprehensive overview, lists every CD8+ T cell epitope that has been published so far.

CIRCUMSPOROZOITE PROTEIN (CSP)

The circumsporozoite protein (CSP) is an important protein of the pre-erythrocytic stage of the parasite. It is the most abundant surface protein on sporozoites and has important roles in the *Plasmodium* life cycle (148). The function of CSP has been investigated in detail in *P. berghei* infection where it is essential for sporulation, gliding mobility and binding to hepatocytes

[1] *PubMed Help* [Internet]. Bethesda, MD: National Center for Biotechnology Information (US); 2005-. Available online at: https://www.ncbi.nlm.nih.gov/books/NBK3827/. Home - PubMed – NCBI; https://www.ncbi.nlm.nih.gov/pubmed/. (Accessed: August 29, 2018).

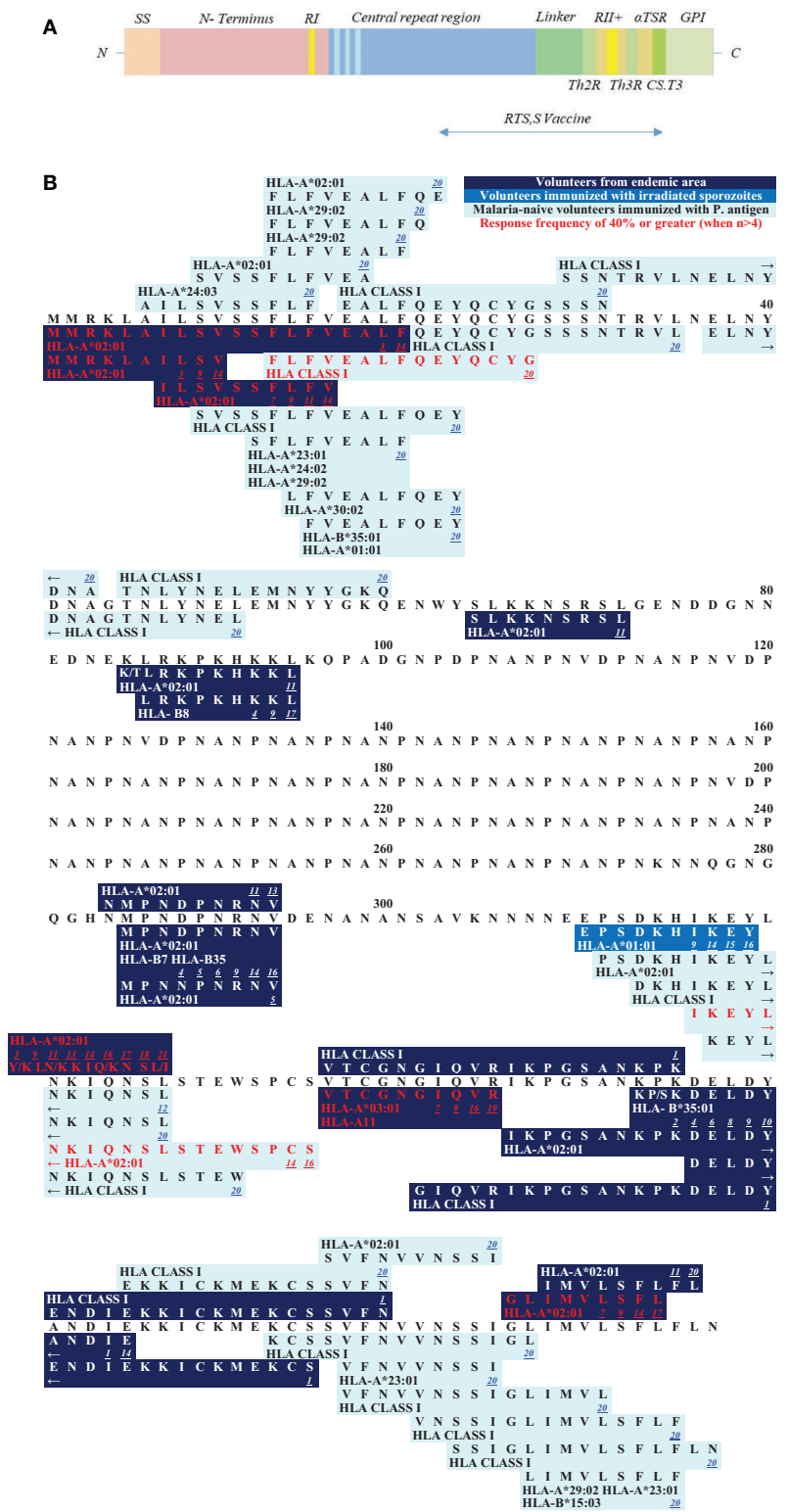

FIGURE 1 | (A) Schematic draft of *P. falciparum* CSP. The signal sequence (SS) and region one (RI) are localized within the N-terminus. The central repeat region is a region of NANP (dark blue) and NVDP (light blue) repeats. The C-terminus (green shades) is linked to the repeat region and contains an α1-helix thrombospondin type-1 repeat (αTSR). The αTSR contains the overlapping Th2-Region (Th2R), region II+ (RII+), Th3- Region (Th3R), and CS.T3. The glycosylphosphatidylinositol (GPI) attachment site is an anchor for the protein to the sporozoite plasma membrane (135, 136). The RTS,S vaccine contains a part of the central repeat region and the C-terminus. **(B)** Epitope map of CSP. All MHC class I epitopes that can be found for the CSP (circumsporozoite protein) are marked in this epitope map (epitopes

(Continued)

FIGURE 1 | longer than 20 AA were excluded). Dark blue boxes: naturally exposed volunteers; blue boxes: volunteers who were immunized with attenuated sporozoites; light blue boxes: malaria-naïve volunteers immunized with *P. falciparum* antigens. Red font color: response frequency of 40% or greater (when more than 4 subjects were tested). The small number next to the epitope is a link to the reference that published the epitope (you will be forwarded to the journal website by clicking onto the link). Within each box the according MHC type is marked and variants within the epitope sequence are indicated by a dash. Most epitopes that were detected within the N-terminus of CSP were found by Sedegah et al. (137). Within the central tandem repeat region no CD8+ T cell epitopes have been found so far. The C-terminus is a very immunogenic region of this protein and contains most of the epitopes.

(149–151). Via CSP, the sporozoites recognize heparan sulfate proteoglycans (HSPGs) which are glycoproteins on the surface of hepatocytes that allow cell interactions (152). The function of CSP in *P. falciparum* malaria is less well studied, but binding to hepatocytes has also been demonstrated (152).

The CSP protein is 397 amino acids in length and shows a similar structure among different *Plasmodium* species, suggesting conserved essential roles for each domain. The protein consists of the N-terminus, the central tandem repeat region and the C-terminus (**Figure 1A**) (153).

The N-terminus, and in particular the first 78 amino acids (AA), is a very well conserved part of the CSP **(Supplementary Figure 1**). It contains region I (RI), a five AA sequence (**KLKQP**) conserved in almost all *Plasmodium* species (154). The N-terminus is important for binding to hepatocytes (152, 155). Cleavage occurs at RI by a parasite protease upon contact with host hepatocytes and leads to a change of an "adhesive" to an "invasive" behavior (156, 157). Antibodies to RI can inhibit cleavage of CSP and therefore impair invasion *in vitro*, as well as *in vivo* (158).

The following epitopes localized within the N-terminus have been described to date (**Figure 1B**): The epitope **MMRKLAILSV** shows a high response frequency (RF) of 40% (6/15) in a study with volunteers from a malaria endemic area (159). The recognition of the peptide was demonstrated via an IFNγ-specific ELISA. It was also recognized by malaria-naïve volunteers immunized with a plasmid DNA vaccine (121, 144). 35.7% (5/14) to 36.4% (4/11) of the volunteers recognized the epitope. The overlapping epitope **MMRKLAILSVSSFLFVEALF** also had a high RF of 78.6% (11/14) in immunized but malaria-naïve volunteers (144, 159)**.** The overlapping epitope **ILSVSSFLFV** showed response frequencies of 50% (1/2) to 71.4% (10/14) in studies with malaria-naïve volunteers who were immunized with CSP, but the epitope could also be detected in inhabitants from a malaria endemic-area [RF: 5.9% (1/17) to 8.3% (3/36)] (74, 121, 141, 144).

Within the N-terminus of CSP Sedegah et al. detected responses directed against 15 CD8+ T cell epitopes in 2013 (137). They performed an IFNγ-specific ELISPOT after adenovirus vector-based immunization of malaria-naïve volunteers with CSP and AMA1. Six to ten subjects were tested, and RF varied from 10% (1/10) to 40% (4/10).

The epitope **LRKPKHKKL** was detected in both, vaccinated malaria-naïve volunteers and volunteers from an endemic area and was restricted to HLA-B8 (63, 121, 145). **LRKPKHKKL** showed a RF of 18.2% (2/11) in a malaria-endemic study population (63).

The tandem repeat region located in the center of the protein is an immunodominant B cell region and most of the described *P. falciparum*-specific antibody-epitopes are located here (160, 161) **(Figure 1A)**. This region comprises of the tetra peptide repeats NANP and NVDP. Notably, no *P. falciparum*-specific CD8+ T cell epitopes have been detected in this region, but several CD4+ T cell epitopes of different length have been found in this area (162–166).

Finally, the C-terminus contains a linker followed by an α-thrombospondin type-1 repeat (αTSR) domain (153, 167) **(Figure 1A)**. Within the αTSR domain the region II-plus is located. This is an 18-amino acid sequence that also mediates adhesion of sporozoites to the heparan sulfate proteoglycans (HSPGs) (168, 169). CSP is anchored to the sporozoite plasma membrane by a glycosylphosphatidylinositol (GPI) attachment site at its C-terminus (170). Lymphoproliferative assays in naturally exposed patients have shown three immunodominant T cell epitope domains located within the C-terminus, called Th2R, Th3R, and CS.T3 (171).

Within the C-terminus of the protein, the following epitopes have been described **(Figure 1B)**:

The epitope **MPNDPNRNV** was tested in malaria-naïve volunteers as well as in subjects from a malaria-endemic area. RF varied from 1/10 (10%) to 1/3 (33%) in malaria-naïve volunteers immunized with CSP (106, 121, 144, 172). The peptide is restricted by different HLA molecules: HLA-A*02:01, HLA-B7, and HLA-B35. Of note, HLA-B7 and HLA-B35 both belong to the HLA-B7 supertype (173). In volunteers from a malaria-endemic area 16.7% (1/6) to 100% (1/1) recognized the peptide (60, 63, 101).

Two epitopes are located within the Th2R region: **EPSDKHIKEY**, restricted to HLA-A*01:01 was only tested in malaria-naïve volunteers who were immunized with plasmid DNA or irradiated sporozoites (106, 121, 144, 174). RF varied from 10% (1/10) to 100% (2/2).

Wang et al. detected the epitope **IKEYLNKIQNSLSTEWSPCS** and RF was 35.7% (5/14) to 100% (8/8) (106, 144). The peptide **Y/KLN/KIQ/KNSL/I** is the best characterized T cell epitope and was described in 8 publications (106, 108, 119, 121, 141, 144, 145, 159, 175). It is also the most polymorph epitope within CSP **(Supplementary Table 1)**. **YLNKIQNSL** was recognized in 23.5% (4/17) volunteers from a malaria-endemic area. In malaria-naïve volunteers who were immunized with a subunit vaccine RF was 13.3 (2/15) to 100% (5/5). **YLKKIKNSL, YLKKIQNSL,** and **KLKKIKNSI** were all detected in a malaria-endemic study population. RF for **YLKKIKNSL** was 60% (9/15) to 100% (1/1), for **YLKKIQNSL**

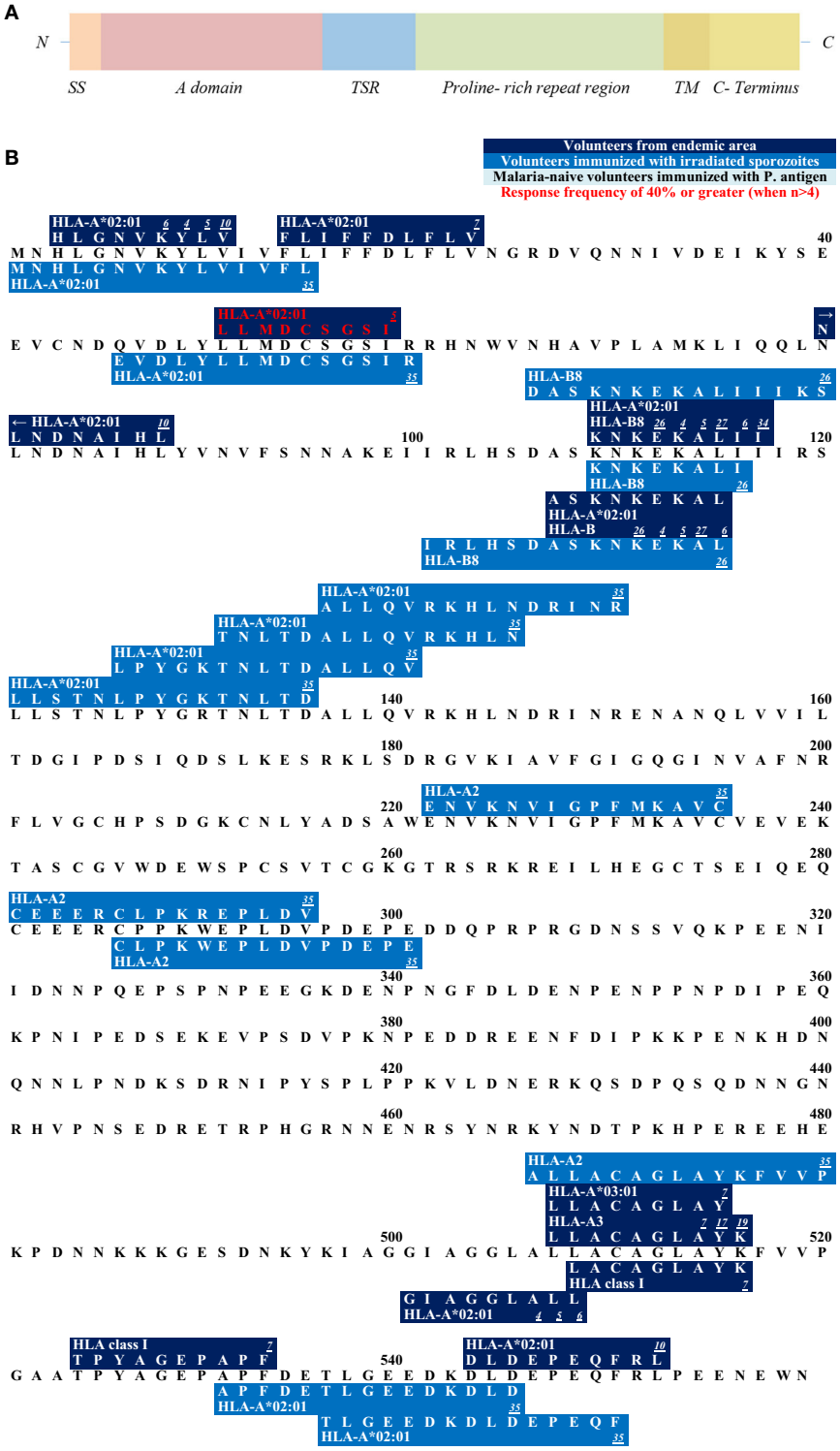

FIGURE 2 | (A) Schematic draft of *P. falciparum* TRAP. This schematic draft shows the signal sequence (SS) at the N- terminus containing two adhesive domains i.e., the A domain (von Willebrand factor A- like domain) and the TSR (thrombospondin type-I repeat) domain (138, 139). This is followed by a proline-rich repeat region, a transmembrane domain (TM) and finally the acidic C-terminus located in the cytosol (140). **(B)** Epitope map of TRAP. Most epitopes within TRAP have been detected in studies that worked with malaria-naïve volunteers who were immunized with attenuated sporozoites.

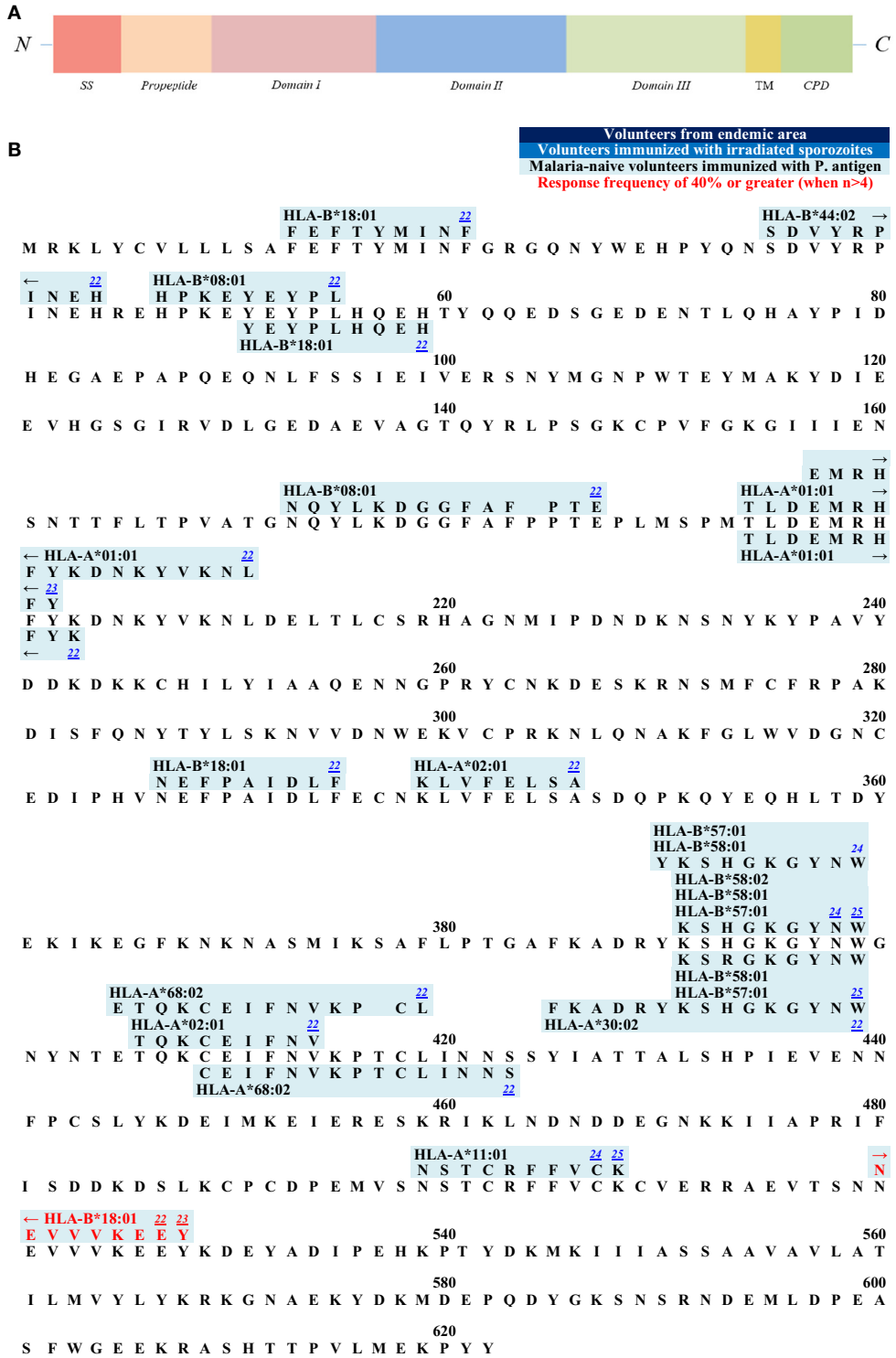

FIGURE 3 | (A) Schematic draft of *P. falciparum* AMA1. This schematic draft shows the signal sequence (SS) toward the N- terminus followed by the propeptide which is cleaved off during the maturing process. Domain I, II, and III build the ectodomain of AMA1 with a hydrophobic cleft and a PAN fold in the tertiary structure. The transmembrane domain (TM) as well as the cytoplasmic domain (CPD) are located within the C-terminus. **(B)** Epitope map of AMA1. Only epitopes that were tested in malaria-naïve study cohorts immunized with *P. falciparum* antigens have been published for AMA1. NEVVKEEY is the only epitope within this antigen that reached a response RF (response frequency) of 40% or greater.

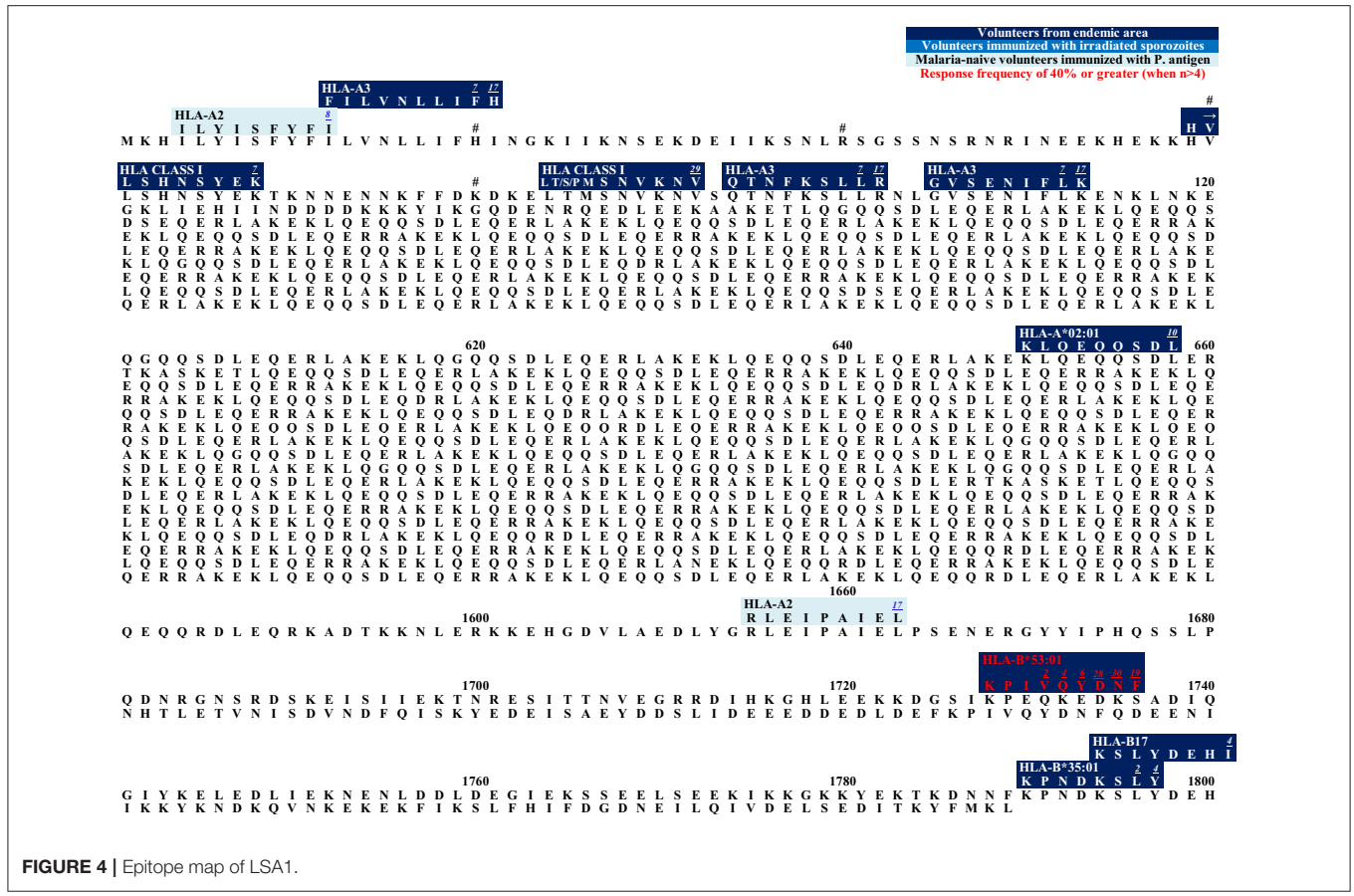

FIGURE 4 | Epitope map of LSA1.

33.3% (5/15) and to **KLKKIKNSI** responded 41.2% (7/17) of the study population. The epitope **VTCGNGIQVR** is located within the region II-plus. This epitope was tested in subunit vaccine trials [RF = 10% (1/10) to 57.1% (4/7)], with irradiated sporozoites [RF = 100% (4/4)] and naturally exposed volunteers [RF = 17.8% (8/45) to 41.7% (5/12)] (74, 102, 121, 144). The epitope is restricted to HLA-A*03:01 and HLA-A*11 (121).

KPKDELDY, restricted to HLA-B*35:01 was detected in malaria-naïve [RF = 100% (1/1)] and naturally exposed subjects (RF = 12.5% (1/8) to 20% (3/15)] (23, 63, 94, 101, 121, 176). **KSKDELDY** had a RF of 10% (1/10) to 12.5% (1/8) in a naturally exposed population.

GLIMVLSFL was tested in subunit vaccine trials [RF = 6.7% (1/15) to 78.6% (11/14)], with irradiated sporozoites [RF = 100% (2/2)] and naturally exposed volunteers [RF = 9.1% (1/11) to 11.1% (4/36)] (74, 121, 145).

The CSP is the most frequently investigated antigen in terms of the number of different studies and number of described T cell epitopes. This is probably due to the fact that CSP was the first of the malarial proteins to be cloned, and as a result, significant efforts have been undertaken to investigate this protein (177). Ever since, CSP has been used as antigen for several vaccine trials so far (12, 178). The RTS,S vaccine contains half of the central repeat region and the entire C-terminus of CSP (**Figure 1A**) (12).

THROMBOSPONDIN-RELATED ANONYMOUS PROTEIN (TRAP)

TRAP is a 559 AA long protein that induces antibody and T cell responses (179). This transmembrane protein is localized in the micronemes (specialized secretory organelles) and on the surface of sporozoites (180). It is important in the infection of hepatocytes (181, 182), has a function during the invasion of mosquito salivary glands, and TRAP also plays a role in sporozoite motility (140).

The N-terminus of TRAP contains the A domain and thrombospondin type-I repeat (TSR) which are two adhesive areas that can bind to heparan sulfate proteoglycans (HSPG) (181, 182) (**Figure 2A**). Upon contact with the host cell, TRAP is released from the micronemes and allows these adhesive domains to interact with the hepatocytes (181–184). Presumably, other non-HSPG-receptors also play a role for hepatocyte invasion (181).

The **C-terminus** of TRAP remains intracellularly located and is attached to the sporozoite motor system (169) (**Figure 2A**). The N-terminus and the intracellular C-terminus form a moving junction and allow gliding without cilia or flagella or major changes in the cell morphology (185, 186).

The N-terminus of TRAP contains 11 different epitopes (**Figure 2B**). **HLGNVKYLV** was tested in HLA-A*02:01 positive

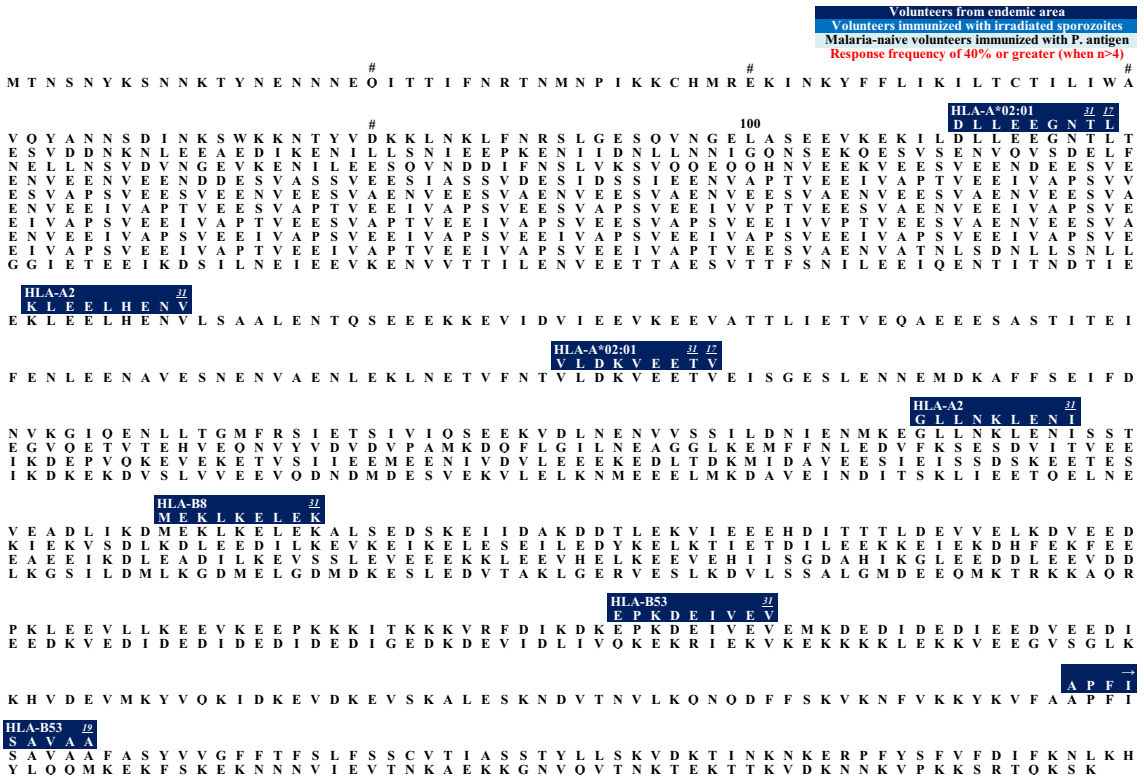

FIGURE 5 | Epitope map of LSA3.

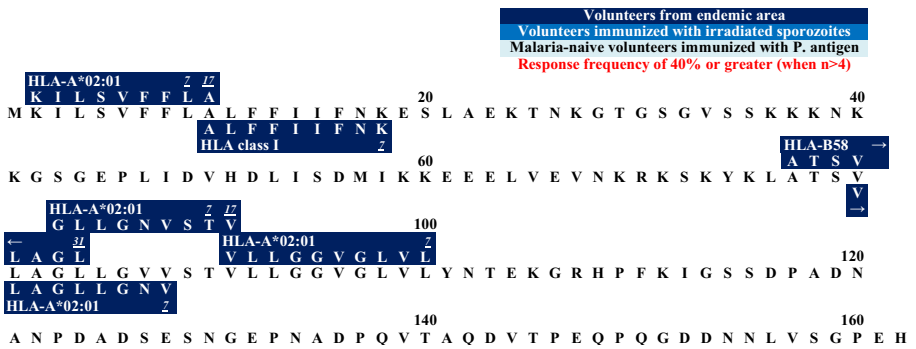

FIGURE 6 | Epitope map of EXP1.

study participants who were naturally exposed to malaria. RF varied from 5.9% (1/17) to 100% (1/1). 11.1% (4/36) of volunteers from a malaria-endemic area and 50% (1/2) of volunteers immunized by irradiated sporozoites responded to the peptide (60, 63, 141).

LLMDCSGSI has shown to be restricted to HLA-A*02:01 and 44.4% (4/9) of volunteers naturally exposed to malaria responded to this epitope (60).

ASKNKEKAL was restricted by HLA-A*02:01 and HLA-B (60, 63, 101, 147, 187) and was recognized by 9.1% (11/1) to 33.3% (2/6) in naturally-exposed volunteers and by 100% (1/1) of volunteers immunized with irradiated sporozoites.

RF scores show that 28.6% (2/7) to 100% (1/1) of subjects from a malaria-endemic area responded to **GIAGGLALL** (60, 63, 101) which is located near the C-terminus. **LLACAGLAYK** was detected in two different study populations: in naturally-exposed volunteers [RF = 22.2% (2/9)] and in study participants who had been experimentally immunized with irradiated sporozoites [75% (3/4)] (74, 145). **LLACAGLAYK** was also detected by malaria-naïve subjects immunized with TRAP [5.3% (1/19)].

APICAL MEMBRANE ANTIGEN 1 (AMA1)

AMA1 is both blood stage as well as a sporozoite stage antigen (22). It is important for invasion of erythrocytes, which makes

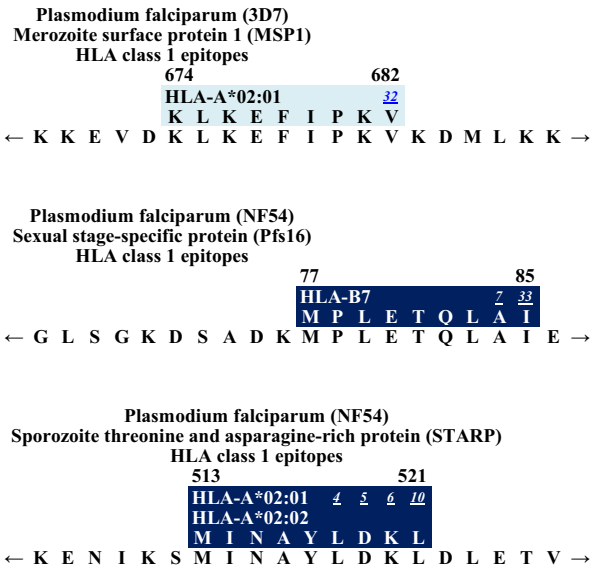

FIGURE 7 | Epitope map of MSP1, Pfs16, and STARP.

AMA1 a target used as blood stage vaccine candidate (22, 188–191). Invasion of erythrocytes is a fast and complex process that takes a series of distinct steps. Firstly, merozoites, the invasive form of *Plasmodia*, attach to the host cell. Secondly, reorientation of the merozoites brings the apical end of the parasite toward the host cell. Finally, the erythrocytes are invaded (192–194).

In order to enter red blood cells (RBC), merozoites release specialized proteins out of secretory organelles called micronemes and rhoptries. AMA1 is one of the proteins, that are released to the surface of merozoites prior to invasion (188, 189, 195). A tight junction between merozoites and RBC is formed with an AMA1 and Rhoptry neck protein complex (RON) (190). The RON protein complex is initially stored within the rhoptries and then inserted into the RBC membrane (22). This junction then allows invagination of the host cell (191).

AMA1 is a 622 AA long protein. At its N-terminus, AMA1 consists of a propeptide that is removed before translocation of the protein onto the merozoite's surface (**Figure 3A**). This cleavage turns the precursor protein (83 kDa) into the mature 62-kDa form. The mature form has structural features of a type 1 integral membrane protein (188, 189, 195).

The ectodomain consists of three domains that is the extracellular part of AMA1 (196). Domain I and II show a high level of sequence conservation across *Apicomplexa*, suggesting a conserved function for these domains, whereas domain III is divergent (197). Domain I and II contain a PAN fold, a flexible loop structure with disulfide bonds. These two domains fold together and form a large hydrophobic cleft on the surface of AMA1 (198). This cleft is speculated to be a ligand-binding pocket (198). It is likely that the flexible and polymorphic loops protect the conserved hydrophobic cleft from detection by the host immune system (197).

All published epitopes on AMA1 were discovered in studies with malaria-naïve vaccines (**Figure 3B**).

The majority of epitopes has been described and published by Sedegah et al. (172). The malaria-naïve study population was immunized with the NMRC-M3V-Ad-PfCA vaccine, which is a combination of two separate recombinant adenovirus 5 constructs, one expressing *P. falciparum* CSP and the other expressing full-length *P. falciparum* AMA1 (both strain 3D7). In this study, the *P. falciparum*-specific CD8+ T cells were detected by ELISPOT. Five subjects were tested, and the response frequencies varied from 20% (1/5) to 40% (2/5). **TLDEMRHFY** and **NEVVVKEEY** are the only CD8+ T cell epitopes that were detected with tetramer-technology after immunization with the NMRC-M3V-Ad-PfCA vaccine (120). **TLDEMRHFY** had a response frequency of 66.7% (2/3) and **NEVVVKEEY** of 100% (1/1). Other epitopes were detected after immunization with DNA plasmids and adeno-viral vectors of CSP and AMA1 (117, 178).

LIVER STAGE ANTIGEN 1 AND 3 (LSA1, LSA3)

The function of liver stage antigen 1 (LSA1) in *P. falciparum* malaria is not clear. The protein, which consists of 1,162 AA (Strain: 3D7) (199), is only expressed during the liver stage and detectable in the parasitophorous vacuole (200). The potential importance of LSA1 as a vaccine candidate was suggested by the observation that the epitope **KPIVQYDNF** restricted by HLA-B53 is associated with resistance to severe malaria (84) (**Figure 4**). RF varied from 23.1% (3/13) to 50% (3/6) in study participants who were naturally exposed to malaria (23, 63, 70, 100–102).

Two epitopes restricted by the protective HLA-B53 alleles have also been found in LSA3 (**Figure 5**): **EPKDEIVEV** (103) and **APFISAVAA** (102). Response frequency of **EPKDEIVEV** was 16.7% (1/6) and **APFISAVAA** showed a RF of 28.6% (2/7). Both epitopes were detected in a naturally exposed study population.

CIRCUMSPOROZOITE-RELATED PROTEIN (EXP1)

Inside the host cell, malaria parasites mature within the parasitophorous vacuole (PV) separated from the cytosol by the PV membrane (PVM) (201). The PVM serves as an interface between parasite and host with important functions like nutrient acquisition, host-cell remodeling, waste disposal, environmental sensing, and protection of the parasite (202). Within the PVM, the circumsporozoite-related protein (EXP1) is a component that is highly expressed during the blood and liver stage (203). Although this protein is likely to be essential, its exact function in the *P. falciparum* malaria life cycle is far from clear. EXP1 is transported out of the parasite into the parasitophorous vacuole membrane (204, 205) after invasion of host cells (206). It is then integrated in the PVM with the C-terminus exposed to the host cell cytosol and the N-terminus in the lumen of the PVM (207). EXP1 has been described to act as a glutathione S-transferase (GST) that protects the parasites from oxidative stress (208) and its enzymatic activity is inhibited by artesunate, a frontline antimalarial drug (208). EXP1 seems also to be essential for

asexual development and the progression across the RBC cycle because the gene is one of the most abundantly transcribed loci during the ring and early trophozoite stages. This is the asexual growth phase inside erythrocytes (209, 210).

EXP-1 is a 162 AA long protein consisting out of a signal sequence (AA 1–23), an N-terminus (AA 23–79), a transmembrane domain (AA 79–101), and a C-terminus (AA 101–162) (211). Six epitopes within this protein have been discovered (**Figure 6**).

All epitopes were detected in subjects from an endemic area. Interestingly, no epitope within the N- or C-terminus has been described so far.

MEROZOITE SURFACE PROTEIN 1 (MSP1)

In the blood phase, the invasion of human erythrocytes is mediated through different proteins. Merozoite surface proteins (MSPs) are thought to be the primary ligands responsible for the low-affinity interactions between the merozoites and the erythrocytes [reviewed in Cowman and Crabb (193)]. The most abundant MSP is the glycosylphosphatidylinositol (GPI)-anchored MSP1 (212). Merozoite surface proteins present themselves as promising vaccine candidates because of their location on the parasite surface and thus exposure to the host immune system. It has been demonstrated that antibodies against MSPs are able to decrease parasitemia *in vivo* (213, 214). Within MSP1, which consists of 1,720 AA (Sequence 3D7) (199), only one CD8+ T cell epitope has been described so far (**Figure 7**): **KLKEFIPKV** is restricted by HLA-A*02:01 and was detected via secreted MHC/mass spectrometry ligand presentation in monochain transgenic mice (143).

SEXUAL STAGE-SPECIFIC PROTEIN (PFS16)

A small proportion of the asexual parasite population enters the sexual pathway and develops into male and female sexual forms called gametocytes, which are essential for transmission to the mosquito vector (215). These gametocytes are inside the parasitophorous vacuole membrane (PVM) and the sexual stage-specific protein (Pfs16), which is 157 AA in length (strain NF54) (199), is part of that membrane and also required for gametocyte maturation (216–219). So far, only one CD8+ T cell epitope has been detected within Pfs16 (**Figure 7**). **MPLETQLAI** had a response frequency of 100% (4/4) in subjects immunized with irradiated sporozoites. It was also tested in volunteers from a malaria endemic area [RF: 6.7% (3/45)—30% (3/10)] (69, 74).

SPOROZOITE THREONINE AND ASPARAGINE-RICH PROTEIN (STARP)

Sporozoite threonine and asparagine-rich protein (STARP) is expressed on the surface of sporozoites that invade hepatic cells, which suggests that it plays a role during parasite entry into the hepatic cell and infection (220). The STARP protein, which consists of 594 AA (strain: 3D7) (199), has been considered to be a potential pre-erythrocytic vaccine candidate because antibodies to this antigen could block *P. falciparum* sporozoite invasion of hepatocytes (221). So far, only one epitope has been located within STARP (**Figure 7**). **MINAYLDKL** is restricted by HLA-A*02:01 and *02:02. All studies that were conducted included subjects from endemic areas. Response frequency varied from 11.8% (2/17) to 75% (6/8) (60, 101, 141).

DISCUSSION

Here we present a comprehensive overview of all human *P. falciparum*-specific CD8+ T cell epitopes published so far, and show their role in the Plasmodium life cycle, potential importance for vaccine development, point out areas where there are still knowledge gaps and outline future directions.

We summarize the data of 132 different *P. falciparum*-specific CD8+ T cell epitopes that have been described in the literature, the respective study population and response frequency, methodology used for detection, and corresponding restricting HLA-molecule.

We hope, that this review will be used as a tool for the selection of suitable epitopes for MHC tetramer synthesis in order to investigate the phenotype of *P. falciparum*-specific CD8+ T cells, for immunomonitoring in vaccine trials, and finally to assist researcher interested in the development of a subunit vaccine. The different and detailed epitope maps give an overview of all epitopes within the antigens (**Figures 1**–**7**). This shows the immunodominant areas of a protein as well as regions that do not elicit a CD8+ T cell immune response.

Notably, the majority of the described *P. falciparum*-specific CD8+ T cell epitopes were discovered in human vaccine trials conducted in malaria-naïve volunteers (121, 145, 172). It has to be taken into account that vaccination with only one *Plasmodium* strain [namely strain 3D7 and NF54 (137, 175, 222)] does not represent a natural malaria infection which can be caused by several strains which might affect the priming and shaping of the T cell response (93, 223). Single-strain vaccines against intense heterogeneous parasite exposure are most likely not going to be successful (15). Further vaccine trials should target more than one antigen from different life-cycle stages to elicit broader immune responses (15, 178). Since to date only a few malaria antigens have been used in vaccine studies, epitopes located within these specific regions are overrepresented in the literature. This demonstrates that the choice of antigens in vaccine trials influences our knowledge about epitopes, but our knowledge about epitopes should also be considered for future vaccine designs. Next to eliciting stronger immune responses, it is widely believed that future malaria vaccines should include a larger number of epitopes in order to show broader protection (15).

In most studies summarized in this review, only small and partly pre-selected (e.g. for HLA-A*2) study populations were tested (an average of 10.3 subjects). Notably, the response frequencies varied between the different study populations. The group that was immunized with irradiated sporozoites had the highest response frequency (72.6%). The group that was immunized with parts of the plasmodial antigens showed a

response frequency of 26.8% and the volunteers from an endemic area had a RF of 16.0% only. The very high RF after immunization with irradiated sporozoites might explain why this is the most effective form of vaccination so far (15).

Only few studies used comprehensive, overlapping peptide libraries for the few antigens tested and mostly already known or *in silico* predicted peptides were utilized (106, 222). Many studies worked with peptide pools and single peptides could not be identified (178, 222). A number of studies focused on HLA-A*2 restricted epitopes, which is the most common expressed Caucasian HLA molecule but not that common in Africa (141, 146). Epitopes recognized in the context of a more diverse set of HLA molecules need to be defined (110).

Furthermore, there is a need for new vaccine targets because most of the known targets have been the same over the last decades (224). For example, only one CD8+ T cell epitope has been defined for an antigen from the sexual stage (Pfs16) (which could potentially prevent transmission by the mosquito) (74). Hill et al. suggest that the search for an association between the ability to respond to a particular malaria antigen or epitope with protective HLA types could be useful to identify an antigen that confers naturally acquired immunity and use it as an antigen for vaccine trials (84).

Indeed, the missing knowledge (and attention paid to) the discovery of new specific malaria epitopes combined with the overall low frequency of *P. falciparum*-specific T cells in the peripheral blood has led to the fact that most immunological studies rather analyzed the function and phenotype of bulk CD8+ T cells (225, 226). Studies on the phenotype and function of *P. falciparum*-specific T cells are largely missing. Fine mapping of the optimal length of the CD8+ T cell epitopes and investigation of their HLA restriction will allow the synthesis of suitable MHC tetramers for phenotypical analysis, most likely by employing further *in vitro* column tetramer-enrichment techniques (227).

The majority of studies on the breadth and specificity of the *P. falciparum*-specific T cell answer was published before 2000 (60, 63, 159). For this and other reasons only few studies used novel and sensitive technologies like ICS, MHC tetramer or even ELISPOT assays (117, 119, 137). Indeed, the last experimental mapping paper was published in 2016 (117, 119). This mirrors the fact that the most intense immunological investigations in the field of malaria epitope discovery took place in the late-1980s and 1990s (110).

Apart from the multicolor flow ICS analysis of vaccine induced *P. falciparum*-specific responses in a couple of vaccine studies, few studies have looked at other effector cytokines or the degree of multifunctionality of antiparasitical cytokines in malaria patients e.g., using multicolor flow cytometry based ICS (228, 229).

As future direction (**Table 2**), mass spectrometry studies e.g., of dendritic cells exposed to malaria parasites, or hepatocytes infected with liver stage antigen in order to identify further protective epitopes will potentially be another highly useful tool for epitope discovery (231).

Interestingly, many of the described *P. falciparum*-specific T cell responses are situated in structurally and functionally important regions. For example: the epitopes that have been described for EXP1, are mostly located within the transmembrane domain but not in the C- nor N-terminus (74, 145). Another example is the epitope **LRKPKHKKL** within the CSP (63, 121, 145) that overlaps with region I, a region which is conserved in almost all *Plasmodium* species and has an important function for hepatocyte invasion (152). Analysis of these and further interactions might be important to assess the role of T cell escape and fitness cost of certain epitopes for future vaccine design.

TABLE 2 | Future directions.

(1) Employment of different *P. falciparum* protein based comprehensive overlapping peptide sets to map the full breadth and specificity of the human malaria-specific CD8+ T cell repertoire.

- Use of sensitive technologies like ELISPOT, ICS.
- Experimental fine mapping of the optimal length and HLA restriction of malaria-specific CD8+ T cell epitopes.
- Screening of large cohorts of patients and vaccines with diverse HLA backgrounds.
- Investigation of the breadth and specificity of the T cell response primed and directed against novel, promising vaccine candidates (e.g., SPECT-1, PFL1620, MALP1.22, PF10925w, PF14_0051 (230).
- Use of "Next Generation Sequencing" for full analysis of the T cell repertoire of malaria patients.
- Construction of novel human MHC class I malaria multimers.
- Further development of mass spectrometry methodologies e.g., of DCs exposed to malaria parasites or hepatocytes infected with liver stages to identify protective epitopes.

(2) Multichannel *ex vivo* phenotypic and functional analysis of malaria-specific T cells using peptide pools, malaria-specific tetramers and employing novel, highly sensitive assays.

(3) Sequencing of circulating *P. falciparum* genome to identify consensus sequences for different malaria antigens, to understand cross-strain immunoreactivity, examine immune pressure, immune escape, and cost of fitness.

(4) Investigation of tissue-resident malaria-specific T cells (e.g., via fine-needle aspiration in the liver).

(5) In iteration based on (1–4) construction and testing of novel multiepitope vaccine constructs containing larger number of antigenic regions.

Supplementary Figures 1–4 depict the entropy blots of the proteins CSP, TRAP, AMA1, and EXP1. This Shannon Entropy-one calculation by the HIV Sequence database compares different sequences of the proteins found on Uniprot, altogether demonstrating the high diversity of the different malaria antigens and strains (199) but also showing the lack of antigen sequencing. For example, only 12 different sequences could be found in open resources for the *P. falciparum* antigen EXP1 (199). Many regions within these antigens used for the entropy map are thought to have important physiological functions but, nevertheless, are polymorph. *P. falciparum*-specific peptides made with a consensus sequence selected out of the sequence of different *P. falciparum* strains could be used for future studies, ideally such a consensus sequence should be designed specifically for individual geographic areas. Studies that investigate the specific CD8+ T cell response should also test different variants from different *Plasmodium* strains if possible. González et al. e.g., tested the variants **TLRKPKHKKL** and **KLRKPKHKKL,** both restricted

to HLA-A*02:01, in 17 subjects (141). RF for **TLRKPKHKKL** was 5.9% whereas for **KLRKPKHKKL** it was 17.6%. This demonstrates that small changes within the AA structure of an epitope can change the binding behavior and improve the priming of CD8+ T cells in context of vaccine design.

Most studies were performed either in the setting of a protective vaccine or in healthy exposed volunteers. Future studies should look at the kinetics of the priming of the CD8+ T cell response during a natural and acute malaria infection with longitudinal long-term follow up to understand the longevity of the *P. falciparum*-specific T cell response. These studies will be crucial to evaluate the difference between vaccine and naturally induced *P. falciparum*-specific T cell responses. Epitope data in the context of clinical stages (age, parasitaemia, complicated, or uncomplicated course of disease, etc.) during an acute disease are lacking because specific CD8+ T cells have rarely been investigated in malaria patients.

Even though a T cell response is mounted against a certain epitope does not mean that this response will correlate with protection. E.g., RAS-vaccines are likely to induce CD8+ T cell responses to many sporozoite proteins, but only a subset of these will be presented by liver stage infected hepatocytes which are the target of protective immunity (232). In different murine and human immunological studies, it could also be demonstrated that only certain epitopes correlate with protection (233–235). The identification of further protective epitopes is therefore necessary. At the same time any future malaria vaccine will have to render a broad population-wide coverage since a protective epitope might be presented by only certain HLA molecules.

Additionally, studies looking at the breadth, specificity, and functionality of tissue-resident, *P. falciparum*-specific T cells in humans are missing. Fine needle aspiration could be introduced as a useful tool to investigate liver-resident T cells because understanding of the CD8+ T cell response from peripheral blood samples has proven to be difficult (236).

The biggest effort in vaccine development was put into the assessment of the humoral response (237). Nevertheless, has this approach not lead to an effective vaccine (e.g., the RTS,S vaccine). The cellular response should therefore not be disregarded (8). On that note, it has become clear that a strong and long lasting CD8+ T cell response is dependent on CD4+ T cells (238, 239). The *P. falciparum*-specific CD4+ T cell response has so far been investigated in more detail with 596 epitopes published compared to the 147 published CD8+ T cell epitopes. This may simply reflect the technical ease of certain assays (lymphoproliferation for CD4+ T cell assessment compared to cytotoxicity assays for CD8+ T cells) but it could also reflect the focus of scientific investigations (110). It is also possible that this disparity is due to biological difficulties in the detection of *P. falciparum*-specific CD8+ T cells (as discussed above).

Further and more comprehensive studies using different cohorts, novel and more sensitive immunological techniques and assays and comprehensive, overlapping peptide sets for a number of different malaria proteins are warranted. In other words: the lacking knowledge of potential *P. falciparum*-specific CD8+ T cell epitopes is hampering optimization for a malaria vaccine.

In summary, the discovery of the full breadth and exact specificities of the *P. falciparum*-specific immune response on an epitope level is of utmost importance for the detailed understanding of the role of CD8+ T cells for control of disease and will give us the tools to optimize vaccine design, immune monitoring of future malaria vaccine trials and to better understand naturally acquired immunity.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

JH, TJ, and JSzW: conception. JH and JSzW: first draft. All authors: important contributions and proofread.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00397/full#supplementary-material

Supplementary Table 1 | *P. falciparum* CD8+ T cell epitopes described in humans. All *P. falciparum*-specific CD8+ T cell epitopes are listed in this table. The reference number (1–35) refers to the number written in each epitope box on the epitope maps. The antigen, the study population, the method used, the response frequency as well as the allele name responding to a certain epitope can be found in this table. Response frequency (RF) represents the number of respondents over the number of subjects tested, when provided by the investigator for the respective assay. ND stands for "not determined" when the investigator did not share this information in the article.

Supplementary Figure 1 | Entropy map of *P. falciparum* CSP. The entropy value was calculated for each AA position. For this calculation 100 different *P. falciparum* sequences were compared. The first 78 aminoacids are well conserved within the N-terminus. The repeat region differs in the number of NANP repeats. The C-terminus is very entropy-rich. We chose the first 100 sequences available on UniProt for this Shannon Entropy-one calculation by the HIV Sequence database.

Supplementary Figure 2 | Entropy map of TRAP. The entropy value was calculated for each AA position. For this calculation six different *P. falciparum* sequences were compared. We chose six sequences available on UniProt for this Shannon Entropy-one calculation by the HIV Sequence database.

Supplementary Figure 3 | Entropy map of *P. falciparum* AMA1. The entropy value was calculated for each AA position. For this calculation 680 different *P. falciparum* sequences of AMA1 were compared. We chose all sequences available on UniProt for this Shannon Entropy-one calculation by the HIV Sequence database.

Supplementary Figure 4 | Entropy map of *P. falciparum* EXP1. The entropy value was calculated for each AA position. For this calculation 12 different *P. falciparum* sequences of EXP1 were compared. We chose all sequences available on UniProt for this Shannon Entropy-one calculation by the HIVSequence database.

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REVIEW
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The Contribution of Co-signaling Pathways to Anti-malarial T Cell Immunity

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Plasmodium spp., the causative agent of malaria, caused 212 million infections in 2016 with 445,000 deaths, mostly in children. Adults acquire enough immunity to prevent clinical symptoms but never develop sterile immunity. The only vaccine for malaria, RTS,S, shows promising protection of a limited duration against clinical malaria in infants but no significant protection against severe disease. There is now abundant evidence that T cell functions are inhibited during malaria, which may explain why vaccine are not efficacious. Studies have now clearly shown that T cell immunity against malaria is subdued by multiple the immune regulatory receptors, in particular, by programmed cell-death-1 (PD-1). Given there is an urgent need for an efficacious malarial treatment, compounded with growing drug resistance, a better understanding of malarial immunity is essential. This review will examine molecular signals that affect T cell-mediated immunity against malaria.

Keywords: malaria, immunity, inhibitory receptors on T cells, stimulatory receptors on T cells, Immuno-therapy, chronic malaria, cerebral malaria, experimental cerebral malaria

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INTRODUCTION

Malaria is a disease caused by parasites of the *Plasmodium spp.* of which there are six species that infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae, P. knowlesi,* and *P. cynomolgi*. Malaria causes serious morbidity in large populations (1) but unlike many other infections, infected individuals do not become resistant to subsequent infections. While adults will develop protection from severe symptoms, chronic, and low grade infections remain a major threat to eradication efforts due to a vast number of carriers. Overall, while tremendous progress has been made in controlling malaria, no vaccine has been completely successful and as such new approaches are required.

Malaria parasites, introduced by mosquitos, first infect the liver and then blood. It is the blood-stage infection that causes the symptoms and lethality associated with malaria. Infants and children in endemic areas are highly susceptible to malaria during the first 5 years but slowly gain resistance to severe, life-threatening infections and then to clinical disease (1). However, sterile immunity is rarely attained (2). As such, there have been well over 100 clinical trials to develop a malaria vaccine and the only one to be approved for use is a pre-erythrocytic vaccine, RTS,S, which comprises of an immunogenic fragment of the circumsporozoite protein of *P. falciparum* with the hepatitis B surface antigen. In field trials, the vaccine showed 30–50% efficacy in the first year following vaccination and this dropped to only 16% in the fourth year, indicating that protective immunity is at some point compromised.

Optimal immune responses against infections require a balance between pro-inflammatory and regulatory immune responses. Pro-inflammatory responses drive protective immunity while regulatory responses control these immune response to prevent tissue damage and also prevent autoimmunity. Immunity against malaria requires a combination of antibodies and T cell responses. Recent research indicates that $CD4^+$ T cells, which consist of several helper-subtypes that shape immune responses, play a much larger role in diminished malarial immunity than previously understood.

Original studies which transferred serum from malaria-protected adults to children, established that antibodies had a critical role in the clearance of parasites (3). However, mouse models have underpinned our understanding of T cell-immunity against malaria. Studies with *P. chabaudi,* which causes a rapidly resolving acute parasitemia followed by multiple recrudescent bouts over many months showed that $CD4^+$ T cells are required to control malaria by their ability to help B cells (4, 5). The resultant antibodies promote parasite clearance by phagocytosis (6). Other studies with *P. chabaudi* showed that $CD4^+$ T cells control the peak of parasitemia in the primary phase of acute blood stage infections (7, 8) via production of high levels of interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α) (9–11). Studies have also shown that IFN-γ and TNF-α cooperate to induce nitric oxide synthase expression in the spleen to control peak parasite burden (12). In contrast, *P. yoelii* YM, *P. yoelii* XL and *P. berghei* ANKA are severe, lethal infections with the last causing cerebral malaria (CM) as this parasite sequesters from the blood into deep tissues including the brain. Studies in mice revealed that $CD8^+$ T cells sequester in the brain during cerebral malaria (13) and with early production of IFN-γ (14) were responsible for mortality. Similarly, $CD8^+$ T cells mediate the loss of marginal metallophilic macrophages and damage to splenic architecture (15). However, several studies have now shown a role for these cells in controlling malaria (7, 16, 17) and more specifically their requirement to control chronic malaria (18) and for long-term sterile immunity (19). These studies suggest that vaccines for blood-stage malaria, would need to target multiple cell types including $CD8^+$ T cells, which has not been undertaken.

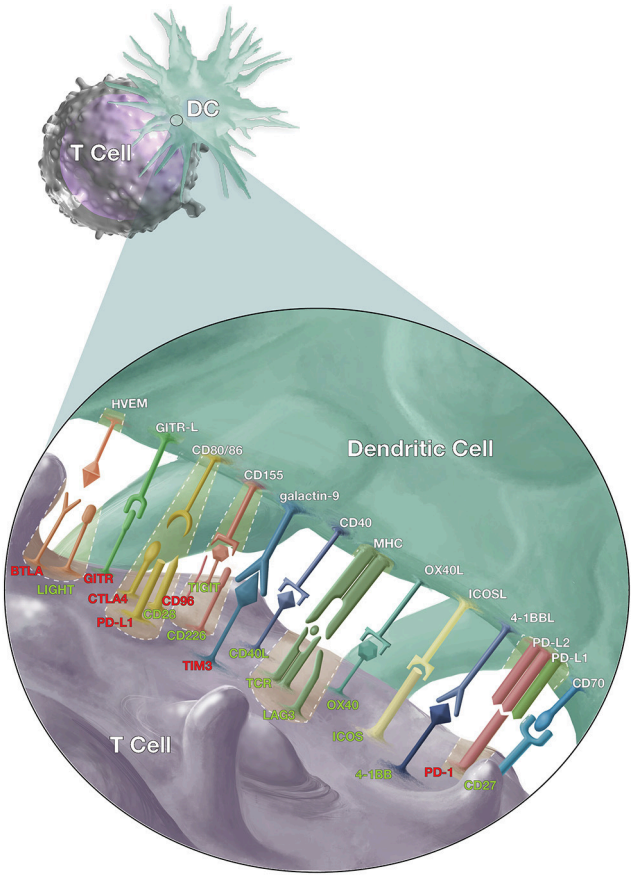

FIGURE 1 | Co-stimulatory and co-inhibitory signals that modulate T cell responses. Dendritic cells (DCs) and T cells interact via several pathways to regulate T cell response against malaria, other pathogens and cancer cells. The T cell receptors (TCR) on antigen-specific T cells first recognize their cognate antigen via the major histocompatibility complex (MHC) molecules on antigen presenting cells. This is followed by the CD28/CD86 interaction, the "second signal," and then a multitude of other signals to fine-tune the immune response. The red labels highlight inhibitory signals while the green show stimulatory signals and cases where inhibitory and stimulatory signals compete for the same ligand or receptor.

The Contribution of Co-signaling Pathways to Anti-malarial Immunity

Signaling between antigen presenting cells [(APCs) including dendritic cells (DC), macrophages, and monocytes] and T cells (**Figure 1**) is a crucial element of adaptive T cell immunity which allows antigen-specific responses to be tightly regulated for effective protection against infections, while minimizing immune-mediated pathology. However, while many of these ligands and receptors are also expressed on other cells (e.g., B cells); in the interest of brevity, this review will focus on signaling between APCs and T cells.

While the interaction of the T cell receptor (TCR) with peptide-loaded major histocompatibility complexes (MHC) on APCs is essential for antigen-specific T cell activation, co-stimulatory (activating), and co-inhibitory (suppressive) molecules determine the magnitude and type of T cell responses. Furthermore, these interactions also co-signal APC to modulate their functions. The requirement for co-stimulation in T cell activation was realized with the discovery of the function of CD28, the canonical co-stimulatory receptor (20, 21). Since then, a large number of co-stimulatory or co-inhibitory receptors, ligands, and counter-receptors have been discovered and studied.

The study of receptor and ligands in the context of infections, cancers and autoimmunity, has revealed that the modulation of co-signaling pathways (also known as "immune checkpoints") underpins the pathogenesis of several diseases. The balance between co-stimulatory and co-inhibitory receptor activation can have a profound effect on disease progression. Recent advances in the understanding of which immune checkpoints influence disease has led to the development of specific targeted immunotherapies that modulate immune functions to alleviate

disease. Most immunotherapies utilize antibodies to modulate immunity and in particular, antibodies against the co-inhibitory receptors cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and PD-1 have been approved by the FDA for treatment of several cancers due to their exceptional efficacy.

Effective immunity against intracellular pathogens requires the development of an optimal T cell response which shows rapid proliferative potential, low apoptosis and poly-functionality (22). During acute infections, optimal functioning T cells clear the pathogen, eventually leading to development of robust memory T cells which have the ability to mount rapid recall response and re-establish poly-functional effector mechanisms upon antigen re-exposure (23). However, some diseases develop "T cell exhaustion," defined by poor effector function, sustained expression of inhibitory receptors (discussed later in detail), poor recall responses and a transcriptional state distinct from that of functional effector or memory T cells (23). There is increasing evidence that these inhibitory receptors affect malarial immunity. As such, the remainder of the review will look at each inhibitory / stimulatory pathway individually and discuss their roles in regulating immunity to *Plasmodium* infection.

Programmed Cell Death Protein-1

PD-1 (CD279) is a member of the extended CD28/CTLA-4 family of T cell regulators (24). PD-1 is expressed on T cells, B cells, natural killer, dendritic cells (DCs) and activated monocytes. The engagement of PD-1 by its ligands, PD-L1 (CD274), and PD-L2 (CD273), normally inhibits T cell functions to induce tolerance and to control the expansion and function of foreign antigen-specific T cell-responses (25–27). PD-1 is not expressed by resting T cells but is induced upon activation (28). PD-1 expression on T cells is up-regulated within 24 h of stimulation and the effects of PD-1 ligation can be seen within a few hours (29). PD-1 needs to be engaged simultaneously with TCR signals to trigger an inhibitory signal (30). PD-1 preserves exhausted T cell populations from overstimulation, excessive proliferation, and terminal differentiation (31). In general, interactions between PD-1 on T cells and its ligand, PD-L1, control the induction and maintenance of peripheral T cell tolerance during normal immune responses and negatively regulate the proliferation and the cytokine production by T cells (32).

Field studies in malaria-endemic areas have shown increased expression of PD-1 on T cells in malaria-infected individuals compared to control subjects, thus implicating PD-1 in immune evasion (33, 34). Significantly, flow cytometric analysis and automated multivariate clustering, has revealed more frequent expression of CTLA-4 or PD-1 on $CD4^+$ T cells from children with complicated malaria compared to uncomplicated malaria (35). PD-1-deficient mice (PD-1KO) rapidly cleared chronic *P. chabaudi* malaria and developed sterile immunity unlike infections in wild type mice (18). Subsequent studies in mice showed that PD-1 also mediated loss of long-term protection against malaria (19). These studies clearly show PD-1 contributes to the pathogenesis of malaria.

PD-L1 and PD-L2, the ligands for PD-1, are expressed on a variety of cells, and their expression on DCs can down-regulate immune responses by T cells (27). Significantly, blockade of PD-L1 signals by antibody, during malaria contributed to improved immunity (33). Furthermore, combined blockade of PD-L1 and Lymphocyte-activation gene 3 (LAG3) immune inhibitory molecules accelerated clearance of non-lethal *P. yoelii* blood-stage malaria by improving $CD4^+$ T cell functions and increasing antibody titres (33). Similarly, blockade of PD-L1 during lethal *P. berghei*-induced experimental cerebral malaria (ECM) also enhanced T cell functions but resulted in an unfavorable outcome in this model, as improved T cell function promoted cerebral disease (36). These studies provide evidence that manipulating checkpoint proteins can improve immune responses.

Other studies of malaria using four mouse models revealed a novel regulatory function for PD-L2 (37). These studies showed that while PD-L1 expressed by DCs down-regulates T cells responses against malaria, PD-L2 protein expressed on DCs improves immune responses by inhibiting PD-L1–PD-1 interactions (37). A therapeutic role for PD-L2 was shown when multimeric form of PD-L2 given to mice infected with lethal malaria, was sufficient to clear the lethal infection and mediate survival from re-infections after several months, without additional treatment (37). Studies of healthy human volunteers before and after infection with experimental *P. falciparum* malaria, found that the expression of PD-L2 inversely correlated with the level of parasitaemia in each individual (37). Overall, this study highlighted the importance of PD-L2 expression for generating malarial immunity.

CD28, CTLA-4, and CD80/CD86

CD28 is a co-stimulatory receptor of the immunoglobulin superfamily, which activates a variety of T cell-activation pathways such as nuclear factor-κB (NF-κB), nuclear factor of activated T cells (NFAT), BCL-XL and mammalian target of rapamycin (mTOR). These signals enhance functions associated with T cell activation such as IL-2 expression, proliferation, survival, and other effector mechanisms.

CD28 was found to be crucial for development of both polyclonal as well as specific antibody responses against malaria, as mice deficient in CD28 had a severe deficit in Ig-production by B cells by day 7 post-infection with *P. chabaudi* compared to WT mice (38). The effects of the CD28 deficit resulted in an inability of these mice to clear parasitemia to sub-patent levels or control re-infections. T cell proliferation was also severely compromised in CD28 deficient mice infected with *P. chabaudi*, although it was not determined whether CD28 directly influenced T cell help, B cell activation and antibody production, or both (38). Another study showed that in $J_H^{-/-}$ mice which lack B cells, a deficiency of CD28 resulted in diminished clearance of *P. chabaudi* parasitemia, demonstrating that CD28 is crucial to both humoral and cell-mediated immunity to malaria (39).

The ligands for CD28 (B7 molecules CD80 and CD86) are expressed by APCs (40). CD80 and CD86 can also bind the inhibitory receptor, CTLA-4 (CD152) which appears on T-cell following activation (41, 42). The mechanism of CTLA-4 function is complex with contradictory reports. CTLA-4 has a higher affinity for CD80 and CD86 than CD28 (43) and is believed to outcompete CD28 for binding to B7 ligands to

regulate early events in T cell activation (44). CTLA-4 is also indicated to directly inhibit TCR signals, reduce IL-2 production and IL-2 receptor expression, and regulate cell cycle progression (44). It was also shown that CTLA-4 can capture B7 ligands from opposing cells by a process of trans-endocytosis resulting in impaired co-stimulation via CD28 (45). CD28 and CTLA-4 binding to CD80 and CD86 thus provide a balance between activation and inhibitory signals. Significantly, PD-L1 expressed by T cells also binds to CD80 on DCs to inhibit T cell activation (46, 47). Importantly, CD28 and PD-L1 on T cells compete for CD80 binding.

Studies using *P. chabaudi* malaria have revealed that antibody blockade of CD80 binding to CD28 did not significantly affect the clearance of parasitemia (48). In contrast, CD86 blockade or dual blockade of CD80 and CD86 resulted in the inability of mice to clear parasitemia to sub-patent levels (48). CD86 or CD80/CD86 blockade increased IFN-γ production while decreasing IL-4 production by *ex vivo* spleen cells (48). *P. chabaudi*-specific IgG1 antibody titres were also reduced by CD86 blockade while early IgG2a titres were increased (48). These effects were not observed with CD80 blockade alone, but were more drastic with dual blockade. This study, along with studies on the role of CD28, indicate that CD86 ligation of CD28 is essential for protective humoral immunity to malaria, while cell-mediated immunity also requires CD28 ligation.

Increased proportions of $CD4^+$ T cells express CTLA-4, OX40, Glucocorticoid-induced TNFR family related gene (GITR), tumor necrosis factor alpha receptor type II (TNFRII), PD-1, LAG3, T-cell immunoglobulin and mucin-3 (TIM3) and CD69 during *P. vivax* (49, 50) and *P. falciparum* (51) malarias. The increased proportion of these cells did not correlate with parasite density in *P. vivax* infections, and did not persist after parasite clearance. However, simultaneous blockade of the CLTA-4, PD-1, and TIM3 signaling restores the cytokine production by antigen-specific cells (50). Significantly, expression of CTLA-4 by T cells is increased in children only during severe malaria (52).

Mouse studies have also shown that lethal malaria induces the production of high levels Transforming Growth Factor- β (TGF-β), which is associated with delayed and blunted IFN-γ and TNF-α responses, failure to clear parasites, and 100% mortality (53). Mechanistic studies showed that cross-linking surface CTLA-4 in cultures of spleen cells taken from mice infected with a lethal infection, induced TGF-β secretion (53). In contrast, blockade of CTLA-4 in mice was found to increase T cell activation and IFN-γ production, resulting in a lower peak parasitemia during non-lethal *P. yoelii* 17XNL infections (54) and could induce cross-species protection against *P. berghei* by inhibition of regulatory T cells (Treg) development (55). Similarly, antibody-mediated blockade of CTLA-4 during *P. berghei*-infection in ECM-resistant BALB/c mice resulted in higher levels of T cell activation, enhanced IFN-γ production, increased intravascular arrest of both parasitised erythrocytes and $CD8^+$ T cells to the brain, and augmented incidence of ECM (36). Given that $CD8^+$ T cells facilitate cerebral disease, CTLA-4 function appears to be protective against CM, as it prevents immune-mediated pathology by restricting T cell activation (54, 56).

Inducible T Cell COStimulator (ICOS)

ICOS (CD278) is a CD28 homolog which regulates $CD4^+$ T cell activation and promotes the induction of $CD4^+$ follicular Th (T_{FH}) cells which support B cell affinity maturation within germinal centers, to generate high-affinity antibodies. When ICOS deficient mice were infected with *P. chabaudi*, primary parasitemia was significantly lower compared to control mice with a corresponding higher frequency of Th1 cells during this early phase of infection (57). $CD4^+$ T cells were capable of expressing PD-1, B cell lymphoma 6, and CXCR5 during early infection, indicating T_{FH} development was not impaired (57). However, capacity to control the chronic phase of infection, which is controlled by $CD8^+$ T cells (18), was impaired without ICOS expression (57) indicating a role for ICOS in $CD8^+$ T cell functions. Anti-ICOS treatment which depleted ICOS expressing $CD4^+$ and $CD8^+$ T cells during a *P. berghei* infection resulted in a concurrent reduction in plasma IFN-γ, confirming the influence of ICOS on Th1 responses (58). Further, although ICOS deficient mice could produce similar titres of parasite-specific antibodies of most IgG isotypes, the affinity of these antibodies was much lower than that of WT mice (57). This study showed that ICOS expression has a deleterious effect on protective Th1 immunity against malaria but is necessary for maintenance of a sustained high-affinity, protective antibody responses. This was confirmed by another study where ICOS expression by $CD4^+$ T-cells, was limited by Interferon-alpha/beta receptor alpha chain (IFNAR1)-signaling to conventional DCs during *P. chabaudi* AS and *P. yoelii* 17XNL infections resulting in hindered resolution of infections, and impaired antibody responses (59).

ICOS expression by T cells may also be involved in the pathogenesis of cerebral malaria as indicated by studies with *P. berghei* which causes ECM in mice. Following *P. berghei* infection, mice with a deletion in the Fc receptor, FcγRIIB, or transgenic mice overexpressing toll like receptor 7 (TLR-7) had lower levels of cerebral pathology than WT mice, but were unable to control parasitemia (60). T cells from FcγRIIB deficient mice had uniformly intermediate levels of ICOS expression, while WT animals had two populations consisting of ICOS-high and–low T cells with the former being associated with better IFN-γ responses. This study indicated that ICOS plays a part in modulating Th1 immunity which supports cerebral pathology in malaria.

HVEM/ /BTLA

The B and T lymphocyte attenuator (BTLA; CD272) and tumor necrosis factor superfamily member 14 (TNFSF14; also known as LIGHT; CD258) compete for interaction with herpesvirus entry mediator (HVEM; also known as tumor necrosis factor receptor superfamily member 14; CD270) and form a part of a complex family of co-signaling molecules. Ligation of LIGHT by HVEM is costimulatory, while BTLA-HVEM binding is co-inhibitory (61, 62). HVEM is expressed by many cells (e.g., hematopoietic, endothelial and epithelial cells), while the LIGHT is expressed on both innate and adaptive immune cells, and BTLA is expressed on naïve T and B cells and is further upregulated on activation.

Following infection of mice with cerebral malaria-causing *P. berghei*, both $CD4^+$ and $CD8^+$ T cells in the spleen exhibited

increased BTLA expression (63). Agonizing this inhibitory receptor with an antibody reduced T cell infiltration in the brain. The T cells that did infiltrate the brain were less activated and produced less inflammatory cytokines. This effect of BTLA on cerebral pathology was only observed if the antibody was administered within the first 2 days of infection, indicating that the effect was due to reduced T cell priming. In contrast, BTLA knockout mice infected with non-lethal *P. yoelii*, exhibited strongly reduced parasitemia and cleared the infection earlier compared with wild-type mice (64). Protection was associated increased pro-inflammatory cytokine production by T cells, without the previously observed pathology (64). Thus, BTLA-HVEM interaction is relevant to malaria but while blockade would be beneficial to controlling parasitemia, it could increase the incidence of cerebral malaria. Of note, genetic variants of BTLA in humans, influence susceptibility to severe chronic hepatitis B (65), which could influence susceptibility to cerebral malaria.

Selective blocking of LIGHT and HVEM signaling does not protect mice from *P. berghei* ECM (66). However, LIGHT also binds to lymphotoxin β receptor (LTβR) besides HVEM (67). Blockade of LIGHT and LTβR signaling pathway early during *P. berghei* infection lead to increased splenic monocytes/macrophages, while blocking later in infection leads to reduced systemic cytokines, thus protecting mice from ECM (66). Thus, indicating that both BTLA and LIGHT are important immune regulators during experimental malaria.

GITR

GITR is another member of the TNFR superfamily (TNFRSF) (68). GITR is expressed in lymphoid tissues and is involved in controlling activated T cells. While resting T cells express low levels of GITR that increase upon activation, $CD4^+CD25^+$ Treg cells express high levels (69). Agonist antibody against GITR enhances anti-CD3-induced proliferation of T lymphocytes, induce proliferation in anergic $CD4^+CD25^+$ Tregs and enhances proliferation of $CD4^+CD25^-$ responder T lymphocytes (70).

Multiple field studies have also implicated Tregs in the pathogenesis of malaria. Specifically, surface expression of GITR and intracellular expression of CTLA-4 were significantly upregulated in Tregs from *P. vivax*-infected individuals, with a positive association between either absolute numbers of $CD4^+CD25^+FoxP3^+GITR^+$ or $CD4^+CD25^+FoxP3^+CTLA\text{-}4^+$ and parasite load (71).

A role for Treg cells in mediating lethality of malaria was shown when mice survived lethal *P. yoelii XL* infections following depletion of Tregs (72). These Tregs were shown to inhibit the activation of effector T cells. However, Tregs did not affect the course of non-lethal *P. yoelii* 17XNL infections (72). Given that GITR is highly expressed by Tregs and supports their suppressive functions, an agonist antibody against GITR was tested during lethal and non-lethal malaria. However, GITR-signaling did not suppress Tregs during the lethal *P. yoelii* XL infection, indicating that Tregs developed a GITR-resistant mechanism during this infection (73).

In contrast, anti-GITR antibodies could partially reverse Treg-functions in *P. berghei* infections (74). Mice infected with *P. berghei* were shown to develop γδ T cells which secrete inflammatory cytokines IFN-γ and IL-17, but Tregs suppress expansion and abolish the effector function of antigen-activated γδ T cells (74). This suppressive effect of Tregs, could be partially reversed by using a monoclonal antibody to GITR. Overall, these studies suggest GITR may control only a subset of Tregs during malaria.

OX40

Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4 or CD134), also known as OX40 receptor, is a co-stimulatory receptor expressed on activated $CD4^+$ and $CD8^+$ T cells as well as a number of other lymphoid and non-lymphoid cells. The ligand for OX40, OX40L, is upregulated on APC following activation. Signals to OX40 promote expansion and survival of antigen-specific T cells (75).

Malaria patients and *Plasmodium*-infected rodents express OX40 predominantly on $CD4^+$ T cells. Treatment of mice with an agonistic antibody against OX40 enhances helper $CD4^+$ T cell activity, humoral immunity, and parasite clearance in during non-lethal malarial infections (76). OX40, is also upregulated on $CD4^+$ and $CD8^+$ T cells in the brain vasculature during ECM (77). Thus, while targeting OX40 may seems to be an attractive means to improve malarial immunity, previous studies showed improving immunity exacerbates ECM (36). Furthermore, agonizing OX40 and blocking PD-L1 during malaria without the complication of ECM, caused excessive IFN-γ responses which inhibited Tfh development required to clear the parasite (76). However, ligation of OX40 during non-lethal *P. yoelii* infection can increase the number of parasite-specific Th1-like memory cells which also exhibit phenotypic and functional features of Tfh cells during recall responses (78). Similarly, targeting OX40 with antibodies in conjunction with vaccination can also improve expansion of antigen-experienced effector $CD8^+$ and $CD4^+$ T cells and commitment protection (79). Overall, OX40 has a complicated function requiring further investigation, especially in the context of malaria.

TIM3

TIM3 (also known as Hepatitis A virus cellular receptor 2), is expressed on $CD4^+$ Th1 and $CD8^+$ T cells. Galectin-9 was suggested to be a ligand for TIM3 (80) although this has been disputed for human T cells (81). TIM3 also binds phosphatidylserine (82). Ligation of TIM3 results in a selective loss of IFN-γ-producing cells and suppression of Th1-mediated autoimmunity (80).

TIM3 expression is significantly increased on a variety of T cells from *P. falciparum*-infected patients (83, 84). There are two main types of γδ T cells found in human blood, Vdelta1 and Vdelta2, and numbers of the latter increase significantly following *P. falciparum* infection in naive adults, but are lost in children following repeated exposure to malaria (83). The secretion of pro-inflammatory cytokines by these T cells was inversely associated with parasitemia and expression of TIM3 (73). Similarly, TIM3 expression is also significantly increased in mice infected with *P. berghei* (84, 85). Experimental models have shown upregulation of TIM3 during malaria leads to

lymphocyte exhaustion which can be reversed with blockade of TIM3, resulting in the accelerated clearance of parasites and relief from the symptoms of *P. berghi*–mediated ECM (84). The role of TIM3 appears to be significant, requiring further investigation.

CD40

CD40 is a costimulatory protein found on APCs, which is required for co-stimulation via CD154 (CD40L) on T cells. As CD40 is an essential component of Th1 and humoral immune responses, studies have looked for polymorphisms which may explain differences in susceptibility to malaria. Field studies in Mali have revealed a marginal susceptibility effect for the CD40L+220C allele from life-threatening malaria (86) but no effect on susceptibility to *P. vivax* in Brazil (87). However, from a functional viewpoint, γδT cells are essential for clearance of the malarial parasites as they have key role in dendritic cell activation via CD40 ligand expression and IFN-γ production (88, 89). As agonistic antibodies to improve immunity are being developed for the treatment of cancer, these could play a role in the future.

CONCLUSION

Malaria is a complex disease and the failure to produce an efficacious vaccine indicates immune suppression mechanisms are impeding protection. The large number of studies cited in this review confirm an important role for immune checkpoint proteins in the pathogenesis malaria. However, the exceptional cost and potential side effects know to occur with immune checkpoint cancer treatments, as well potential exacerbation of cerebral malaria, has impeded any attempt to progress such treatments for malaria. Nevertheless, given rising drug resistance, difficulties in generating an efficacious vaccine for malaria and that some treatments such as soluble PD-L2 generate long-term protection with a reduction in the incidence of cerebral malaria, indicate that checkpoint blockade needs to be further explored.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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NK1.1 Expression Defines a Population of CD4$^+$ Effector T Cells Displaying Th1 and Tfh Cell Properties That Support Early Antibody Production During *Plasmodium yoelii* Infection

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Early plasmablast induction is a hallmark of *Plasmodium* infection and is thought to contribute to the control of acute parasite burden. Although long understood to be a T-cell dependent phenomenon, regulation of early plasmablast differentiation, however, is poorly understood. Here, we identify a population of CD4$^+$ T cells that express the innate NK cell marker NK1.1 as an important source of T cell help for early plasmablast and parasite-specific Ab production. Interestingly, NK1.1$^+$ CD4$^+$ T cells arise from conventional, naive NK1.1$^-$ CD4$^+$ T cells, and their generation is independent of CD1d but critically reliant on MHC-II. CD4$^+$ T cells that express NK1.1 early after activation produce IFN-γ and IL-21, and express the follicular helper T (Tfh) cell markers ICOS, PD-1 and CXCR5 more frequently than NK1.1$^-$ CD4$^+$ T cells. Further analysis of this population revealed that NK1.1$^+$ Tfh-like cells were more regularly complexed with plasmablasts than NK1.1$^-$ Tfh-like cells. Ultimately, depletion of NK1.1$^+$ cells impaired class-switched parasite-specific antibody production during early *Plasmodium yoelii* infection. Together, these data suggest that expression of NK1.1 defines a population of rapidly expanding effector CD4$^+$ T cells that specifically promote plasmablast induction during *Plasmodium* infection and represent a subset of T cells whose modulation could promote effective vaccine design.

Keywords: malaria, T follicular helper cell, antibody, NK1.1, plasmablasts

INTRODUCTION

Despite decades of research, a highly efficacious vaccine against the protozoan parasite *Plasmodium* has yet to be developed, and malaria continues to remain a significant global health problem (1). Although resistance from severe disease is mediated in part by parasite-specific Abs, protective anti-*Plasmodium* Abs are slow to develop in humans and challenging to induce artificially (2). Moreover, a clear understanding of why Ab-mediated immunity is slow to develop is still lacking. Vaccine failure has been attributed to antigenic variation and genetic polymorphisms within the *P. falciparum* (the predominant disease-causing parasite of humans) genome as a whole, as well the parasite's ability to modulate expression of essential parasite proteins such as PfEMP-1 (3). These

factors, as well as others employed by the parasite, lend credence to the idea that *P. falciparum* subverts B cell responses in a manner that results in the inefficient acquisition of protective Abs (2). Thus, further insight into how *Plasmodium* infection shapes the subsequent immune response, including its impact on T and B cell differentiation, could lead to novel vaccine strategies designed to stimulate the production of high affinity, parasite-specific Abs.

Recently, glycolipid-reactive CD4$^+$ NKT cells were evaluated in numerous vaccine platforms (including anti-malarial strategies such as irradiated sporozoite vaccination) due to their adjuvant potential (4, 5). NKT cells are a distinct T cell subset that express NK cell markers, intermediate levels of αβ-TCRs, and a biased repertoire of Vα and Vβ chain genes that bind lipid antigens presented in the context of the MHC class-I like molecule CD1d (abundantly expressed on professional APCs such as B cells and dendritic cells). The adjuvant potential of NKT cells is primarily based on their ability to rapidly respond to antigenic stimulation by secreting IL-4 and IFN-γ, which results in the activation of numerous immune cells, including dendritic cells, NK cells, B cells and CD4$^+$ and CD8$^+$ T cells (5–7).

In the context of malaria, many sporozoite and merozoite surface-localized proteins are GPI anchored. *P. falciparum* GPI can be loaded and presented on CD1d *in vitro*, resulting in the activation, proliferation, and production of IL-4 by NKT cells (8). However, the role of NKT cells in the immune response against *Plasmodium in vivo* is controversial, particularly with regard to blood stage infection. For example, CD1d-deficient mice mount a diminished Ab response during blood-stage *P. berghei* ANKA infection (9), but no difference in parasitemia or survival was noted in *Cd1d*$^{-/-}$ mice infected with *P. yoelii* or *P.c. adami* (10, 11). Nevertheless, the identification of CD1d-independent NKT cells (7, 12) suggests subsets of conventional MHC-restricted T cells may also adopt NK-like characteristics, and potentially participate in anti-malarial immunity. For example, CD1d-independent innate-like CD8$^+$ T cells were recently identified (13, 14). Furthermore, innate NK-like phenotypic characteristics were just observed in B cell subsets (14). As a whole, these studies suggest a variety of adaptive immune cells can adopt innate NK-like characteristics to accelerate, modify, or regulate conventional adaptive immunity.

Thus, as an alternative means to promote or enhance Ab production, we sought to assess the role of non-conventional, innate-like CD4$^+$ T cells in the humoral response during murine *Plasmodium* infection. Here, we describe a population of CD1d-independent MHC-II-restricted NK1.1-expressing CD4$^+$ TCRβ^{hi} T cells that expand dramatically during acute *P. yoelii* infection. NK1.1-expressing CD4$^+$ T cells produced IFN-γ and IL-21 more abundantly than their NK1.1$^-$ counterparts. Interestingly, this population showed a higher frequency of ICOS, PD-1, CXCR5 and Bcl6 expression—markers associated with Tfh cell differentiation—than non-NK1.1–expressing CD4$^+$ T cells. Thus, NK1.1-expressing CD4$^+$ T cells constituted a significant proportion of the early Tfh-like cell response. Strikingly, these Tfh-like NK1.1$^+$ cells were found complexed with plasmablasts more frequently than their NK1.1$^-$ counterparts.

Depletion of NK1.1-expressing T cells led to dramatically fewer class-switched parasite-specific Ab-secreting cells during the first week of infection, and a concomitant decrease in serum MSP-1$_{19}$—specific Abs. Thus, infection-induced NK1.1$^+$ T cells represent a T cell population critically poised to provide B cell help and promote rapid Ab production during *Plasmodium* infection.

MATERIALS AND METHODS

Mice

C57BL/6J, *Cd1d1-d2*$^{-/-}$, *H2dIAb1-Ea*$^{-/-}$, *Cd19*$^{tm1(cre)Cgn}$ *Igh*^{b}, B6.SJL-Ptprca Pepcb/BoyJ, and *Ighm*^{tm1Cgn} (μMT) mice were obtained from The Jackson Laboratory. Male BALB/c mice were purchased from Charles River Laboratories. Female mice between the ages of 7–9 weeks were used for all experimental cohorts. Experimental results were confirmed in similarly aged male mice to ensure that the experimental results displayed no sexual bias. Male BALB/c mice were used for routine passage of parasites, as previously described (15). All mice were housed and bred in specific-pathogen-free facilities at the University of Arkansas for Medical Sciences in accordance with institutional guidelines. The IACUC at the University of Arkansas for Medical Sciences approved all procedures on mice in this study. All animal procedures were performed in compliance with the Animal Welfare Act and accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011.

Parasites

Plasmodium yoelii 17XNL and *Plasmodium chabaudi chabaudi* AS infections were performed as previously described (15, 16). Briefly, cryopreserved parasite stocks were intraperitoneally (i.p.) inoculated into male BALB/c mice; at peripheral blood parasitemia of approximately 1–2%, mice were sacrificed, and blood was collected into heparinized RPMI. 10^5 parasitized erythrocytes were subsequently i.p. injected into experimental mice to establish infection. All procedures involving *Plasmodium yoelii* 17XNL and *Plasmodium chabaudi chabaudi* AS were approved by the IBC at the University of Arkansas for Medical Sciences. All procedures were performed in compliance with the guidelines outlined in the 5th edition of "*Biosafety in Microbiological & Biomedical Laboratories*," U.S. Department of Health and Human Services, 2009.

Flow Cytometry

Single cell suspensions were obtained by passing spleens through 40-micron filters; following ammonium chloride erythrocyte lysis; cells were re-suspended in complete RPMI (RPMI 1640 supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamate, 1% penicillin-streptomycin, and 0.1% β-mercaptoethanol). Before FACs staining, Fc receptors on splenocytes were blocked with normal rat and mouse serum, and 2.4G2 Abs. For surface staining, Ab cocktails were re-suspended in FACs staining buffer containing 0.2% BSA and 0.2% 0.5M EDTA in PBS, followed by fixation in 4% paraformaldehyde

(PFA). For intracellular cytokine staining, splenocytes were first stimulated for 4 h at 37°C in the presence of PMA, ionomycin and Brefeldin A. Cells were then surface stained and fixed with 4% PFA. Intracellular staining was performed following permeabilization with 0.1% saponin dissolved in FACs buffer. For nuclear transcription factor and Ki-67 staining, cells were first surface stained as described, followed by simultaneous fixation and permeabilization with a FoxP3 fix/perm kit per manufacturers direction (ThermoFisher Scientific). Annexin V staining was performed according to the manufacturer's instructions in the Annexin V-FITC apoptosis detection kit (ThermoFisher Scientific). To assess T-B cell interactions, FACs staining was performed as described earlier with the exception that EDTA was withheld from all staining buffers to prevent disruption of cell-cell interactions (16).

Fluorescently labeled Abs were purchased from BioLegend, ThermoFisher Scientific, or BD Biosciences. Abs used for flow cytometry include CD4, TCRβ, NK1.1, PD-1, CXCR5, CD44, CD62L, ICOS, CD8α, Annexin V, Ki-67, Ly6C, T-bet, Bcl6, Gata3, RORγt, Foxp3, IFN-γ, TNF, IL-17, CD11a, CD138, B220, CD45.2, PD-L1, IgM, CD45, Ter119, CD11c, CD11b. Fixable viability dye and propidium iodide were from ThermoFisher Scientific. For IL-21 staining, recombinant mouse IL-21 receptor fused to human Fc (R & D Systems) staining was performed first, followed by secondary anti-human Fc-PE Ab (ThermoFisher Scientific) staining. The PBS-57 analog of α-galactosylceramide loaded CD1d tetramer and unloaded CD1d negative control were acquired from the NIH Tetramer Core Facility at Emory University. Samples were acquired on a BD Biosciences LSRFortessa and analyzed using FlowJo version X software.

ELISA

Relative parasite-specific Ab titer was assayed by coating Immulon HBX 4x plates (Thermo Scientific) with *P. yoelii* MSP-1_{19} recombinant protein or whole parasite lysate. Serially diluted serum was applied followed by incubation with HRP-conjugated IgM, and IgG-specific Abs (Southern Biotech). ELISAs were developed with SureBlue substrate (KPL), and the absorbance of each well was measured at 450 nm on a FLUOstar Omega plate reader (BMG Labtech).

Parasitemia

Peripheral blood parasitemia was assessed by flow cytometry, as recently described (17). Briefly, ∼1 uL of blood was obtained by tail vein puncture and immediately re-suspended in heparinized PBS. RBCs were washed with FACs buffer (described above) and stained with Hoechst 34580 (Life Technologies) to detect DNA, APC-labeled CD45 (to exclude lymphocytes), and PerCp-Cy5–labeled Ter119 (to label erythrocytes). Cells were obtained on a BD Biosciences LSRFortessa, and parasitemia was assessed with FlowJo version X software.

Adoptive Transfer

Naïve ($CD44^{lo}CD62L^{hi}$) $CD4^+$ T cells sorted from C57BL/6J mice were transferred i.v. through retro-orbital injection into congenic CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ) mice. On the following day, recipient mice were infected i.p. with 10^5 *P. yoelii* infected RBCs. Transferred cells were recovered from infected mice on day 7 and 11 p.i. utilizing anti-CD45.2-PE, anti-PE microbeads (Miltenyi Biotech) and positive selection on an autoMACs Pro Separator (Miltenyi Biotech).

Antibody Depletion

For depletion of NK1.1-expressing cells, 200 μg of anti-NK1.1 (clone PK136, BioXcell) or isotype control mouse IgG2a (BioXcell) was i.p. injected every other day, beginning on day−1 of *P. yoelii* infection.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). Specific tests of statistical significance are detailed in the figure legends.

RESULTS

$NK1.1^+CD4^+$ T Cells Expand Dramatically During Acute *P. yoelii* Infection

To identify NKT cells during infection of wild-type (WT) mice with *P. yoelii* 17XNL—a non-lethal, self-resolving rodent model of malaria—splenocytes were stained for CD4, TCR-β, and NK1.1. Although a distinct population of NKT cells ($CD4^+$ TCR-$\beta^{int}NK1.1^+$) was present in the spleen of naïve WT mice, this population did not expand during infection. Instead, a TCR-$\beta^{hi}NK1.1^+$ population of $CD4^+$ T cells emerged in the spleen as early as day 5 post infection (p.i.) (**Figure 1A**), and this population phenotypically resembled NK1.1-expressing T cells previously described in the spleen following infection with *P. yoelii* sporozoites (10). Similarly, TCR-$\beta^{hi}NK1.1^+$ $CD4^+$ T cells expanded in the spleen after *P. chabaudi* infection (**Supplemental Figure 1A**), a rodent model characterized by persistent low-level parasitemia. Additionally, NK1.1 expression was not limited to $CD4^+$ T cells, as splenic $CD8^+$ T cells also upregulated this marker after *P. yoelii* infection (**Supplemental Figures 1B,C**).

TCR-$\beta^{hi}NK1.1^+$ $CD4^+$ T cells expanded rapidly and remained elevated until peak parasitemia (day 14 p.i.) but contracted significantly upon resolution of the infection at day 24 p.i. (**Figures 1B,C**). TCR-$\beta^{hi}NK1.1^+$ $CD4^+$ T cells also contracted during the chronic stage of *P. chabaudi* infection (**Supplemental Figure 1A**). By day 8 p.i. the majority of the TCR-$\beta^{hi}NK1.1^+CD4^+$ T cells exhibited a $CD44^{hi}CD62L^{lo}$ phenotype indicative of activated effector T cells (**Figure 1D**) and maintained this phenotype over the course of infection (**Figure 1E**). Not surprisingly, a significantly smaller percentage of $NK1.1^-CD4^+$ T cells displayed a $CD44^{hi}CD62L^{lo}$ phenotype during the active infection (**Figure 1E**), as the majority of these cells maintain a naïve phenotype. Also, TCR-$\beta^{hi}NK1.1^+CD4^+$ T cells showed a significantly higher fold expansion in total cell numbers between day 5 and 8 p.i. compared to $NK1.1^-$ $CD4^+$ T cells. By day 8 p.i., similar numbers of activated ($CD44^{hi}CD62L^{lo}$) $NK1.1^-$ and $NK1.1^+$ $CD4^+$ T cells were seen over the course of the infection except for day 24 when a significant reduction in $NK1.1^+$ cell numbers occurred with no corresponding loss of $NK1.1^-$ cells (**Figure 1F**). As

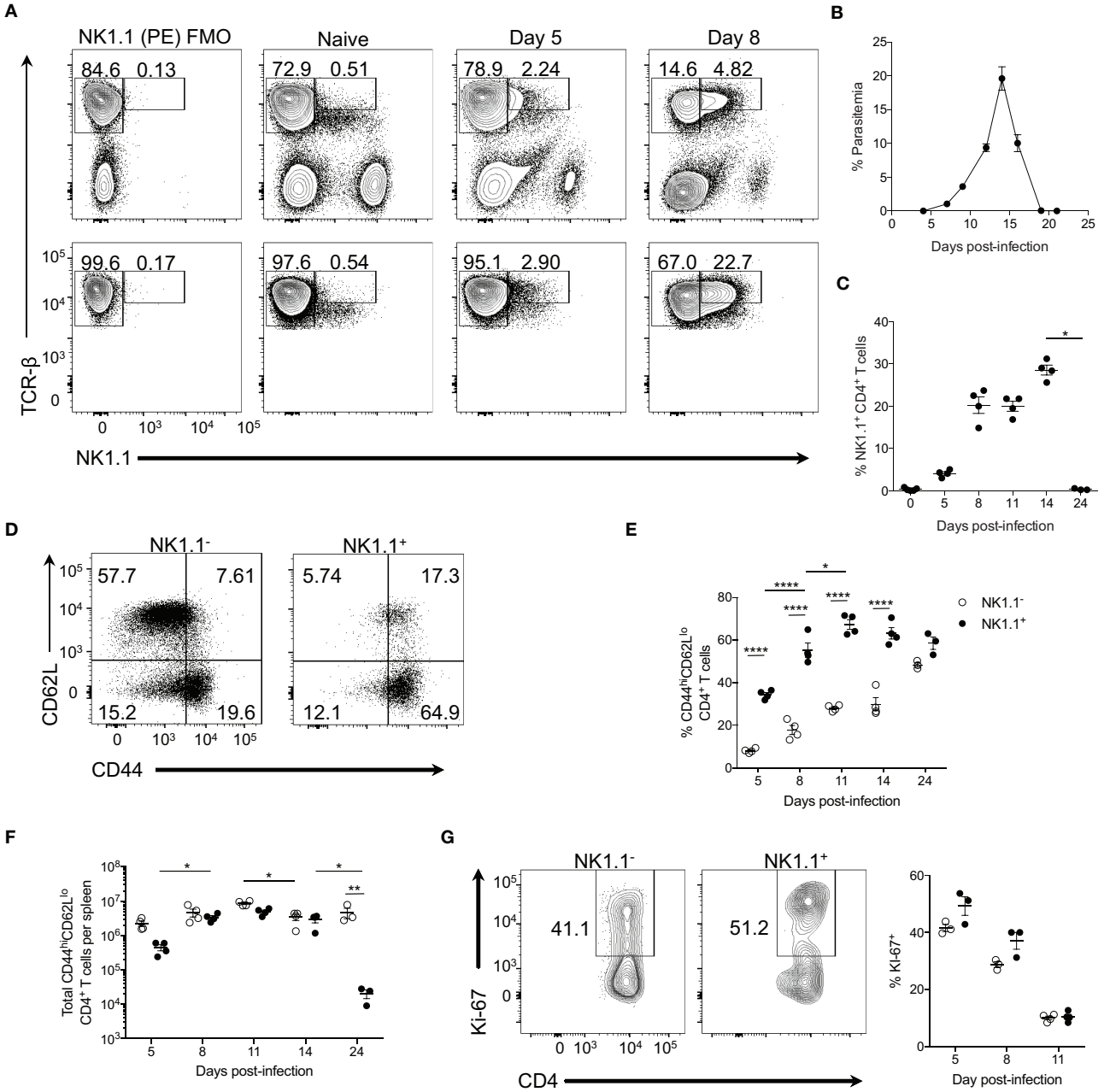

FIGURE 1 | NK1.1$^+$ CD4$^+$ T cells expand during acute *P. yoelii* infection. **(A)** Top: Representative TCRβ and NK1.1 expression at days 5 and 8 post-*P. yoelii* infection. Gated on live splenocytes. Bottom: Representative plots that were previously gated on live, CD4$^+$TCRβ$^+$ splenocytes showing TCRβ and NK1.1 expression at days 5 and 8 post-*P. yoelii* infection. FMO, fluorescence minus one staining control. **(B)** Peripheral blood parasitemia as determined by FACs. **(C)** Frequency of NK1.1 expression among live CD4$^+$TCRβ$^+$ T cells. Significance assessed by one-way ANOVA Kruskal-Wallis test with *post-hoc* Dunn's multiple comparisons test. *$p <$ 0.05. **(D)** Representative CD44 and CD62L expression among live, NK1.1 positive and negative CD4$^+$TCRβ$^+$ splenocytes at day 8 post-infection. **(E)** Frequency of CD44^{hi}CD62L^{lo} of live CD4$^+$TCRβ$^+$ T cells between days 5 and 24 post-infection. An aligned rank transformation was performed on non-parametric data before determining significance by two-way ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. *$p < 0.05$, ****$p < 0.0001$. **(F)** Total number of NK1.1$^-$ or NK1.1$^+$ CD4$^+$TCRβ$^+$ T cells per spleen. An aligned rank transformation was performed on non-parametric data before determining significance by two-way ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. *$p < 0.05$, **$p < 0.01$. **(G)** Representative Ki-67 staining and frequency of expression among live, NK1.1 positive and negative T cells at day 8 post-infection. Cells previously gated on live, CD44^{hi}CD62L^{lo}CD4$^+$TCRβ$^+$ splenocytes. Data are representative of two **(G)** or three **(A–F)** independent experiments (error bars, s.e.m.).

TCR-β^{hi}NK1.1$^+$CD4$^+$ T cells expanded more rapidly than NK1.1$^-$CD4$^+$ T cells, we sought to more closely assess their proliferation. At day 5 and 8 post-*P. yoelii* infection, we observed a higher frequency of Ki-67 expression (a nuclear antigen associated with cell cycle progression) in activated (CD44^{hi}CD62L^{lo}) TCR-β^{hi}NK1.1$^+$CD4$^+$ T cells relative to NK1.1$^-$CD4$^+$ T cells (**Figure 1G**). Collectively, these data indicate that TCR-β^{hi}NK1.1$^+$CD4$^+$ T cells represent a population of highly activated and proliferating effector CD4$^+$ T cells that arise in the spleen during acute *P. yoelii* infection.

NK1.1$^+$CD4$^+$ T Cells Are MHC-II-Restricted and CD1d-Independent

NK1.1 expression on CD4$^+$ T cells is typically utilized for the identification of NKT cells. Therefore, we sought to determine if TCR-β^{hi}NK1.1$^+$ CD4$^+$ T cells were indeed a population of NKT cells. Type I NKT cells represent the most well-classified subset of NKT cells and express the invariant Vα14-Jα18 TCR-α chain complexed to either a Vβ8.2, Vβ7, or Vβ2 TCR-β chain in mice, facilitating binding to glycolipid antigens displayed by the major histocompatibility (MHC) class I–like molecule CD1d (7). To assess the expansion of type I NKT cells in the spleen after *P. yoelii* infection, PBS-57-loaded (an analog of the CD1d binding glycolipid α-galactosylceramide) CD1d-tetramer was used. Although tetramer-binding TCRβ$^+$ NKT cells are identifiable in the spleen after infection (**Figure 2A**), no significant increase in type I NKT cell numbers occurred throughout the infection (**Figure 2B**). Further examination of the tetramer-positive cells revealed that these cells are a heterogeneous population of TCR-β^{int} NK1.1$^-$ and NK1.1$^+$ NKT cells and not the TCR-β^{hi}NK1.1$^+$ CD4$^+$ T cells that emerge after infection (**Figure 2A**). These data indicate that type I invariant NKT cells do not account for the increase in NK1.1-expressing CD4$^+$ T cells observed during acute *P. yoelii* infection.

While CD1d tetramers are capable of identifying type I NKT cells, type II NKT cells are not as easily detected, despite similar CD1d restriction (7). Thus, to determine if the NK1.1$^+$CD4$^+$ T cells observed here are type II NKT cells, WT, CD1d-deficient (lacking type I and II NK-T cells), and MHC-II-deficient (lacking conventional CD4$^+$ T cells) mice were infected with *P. yoelii.* As expected based on previous findings (10, 18) class II-deficient mice were unable to control peak parasitemia, while *Cd1d1-d2*$^{-/-}$ mice showed higher parasitemia that peaked on day 18 compared to day 16 in WT mice, but they resolved the infection within the same time frame as WT mice (**Supplemental Figure 2A**). Interestingly, the absence of CD1d did not impair the expansion of TCR-β^{hi}NK1.1$^+$ CD4$^+$ T cells, but the lack of MHC-II expression ablated induction of this subset (**Figures 2C,D**). This data indicates that NK1.1-expressing T cells are derived from a population of CD4$^+$ T cells that bind protein antigen presented in the context of MHC-II. Importantly, NKT cells (TCR-β^{int}CD4$^+$ NK1.1$^+$) are present in all three genotypes of mice following infection, including *Cd1d1-d2*$^{-/-}$ mice; this latter population most likely represents previously described CD1d-independent NKT cells (7). To demonstrate that TCR-β^{hi}NK1.1$^+$ T cells are derived from conventional CD4$^+$ T cells that do not express NK1.1 naïve NK1.1$^-$CD4$^+$ T cells were sorted from uninfected donor mice and transferred into congenic mice followed by infection of recipient mice with *P. yoelii* 1 day after transfer. Activated donor and endogenous CD4$^+$ T cells expressed NK1.1 at a similar frequency on day 7 p.i., indicating that TCR-β^{hi}NK1.1$^+$CD4$^+$ T cells are indeed derived from NK1.1$^-$CD4$^+$ T cells (**Figure 2E**).

To further alleviate the concern that the observed NK1.1 staining was an artifact of flow cytometry staining (i.e., non-specific labeling), we also assessed the induction of TCR-β^{hi}NK1.1$^+$CD4$^+$ T cells in *P. yoelii*-infected BALB/c mice, which do not express the protein NK1.1. As expected, unlike in C57BL/6 mice, we did not identify TCR-β^{hi}NK1.1$^+$CD4$^+$ T cells after *P. yoelii* infection in BALB/c mice. Additionally, we did not observe staining with the isotype control Ab IgG2a in C57BL/6 or BALB/c mice, further validating the accuracy of NK1.1 staining (**Supplemental Figure 3**). Together, these data suggest that infection-induced NK1.1-expressing CD4$^+$ T cells are not type I or II NKT cells but are instead a subset of conventional MHC-II-restricted CD4$^+$ T cells, hereafter referred to as NK1.1$^+$ CD4$^+$ T cells.

NK1.1$^+$CD4$^+$ T Cells Express Transcription Factors Associated With the Th1 and Tfh Cell Lineage That Are Poised to Produce Cytokines Related to These Subsets

To determine if NK1.1$^+$ CD4$^+$ T cells differ phenotypically from their NK1.1$^-$ counterparts we first assessed their ability to express T-bet and Bcl6, transcription factors associated with T helper type 1 (Th1) and follicular helper T (Tfh) cell differentiation respectfully. Four primary populations emerged from this analysis: Tbet$^+$Bcl6$^-$, Tbet$^-$Bcl6$^+$, Tbet$^+$Bcl6$^+$, and T cells that did not express either Tbet or Bcl6, all of which were apparent at day 8 and 11 p.i. (**Figure 3A**, **Supplemental Figure 4** and data not shown). Although all four of these populations existed within the activated (CD44 hiCD62L^{lo}) NK1.1$^+$ and NK1.1$^-$ pools, a significantly higher percentage of the NK1.1$^-$ T cells expressed Tbet at day 8 and 11 p.i., while the frequency of Bcl6 expression amongst NK1.1$^+$ T cells was significantly higher at day 8 and 11 p.i. No difference in the percentage of Tbet$^+$Bcl6$^+$ cells was seen between NK1.1$^+$ and NK1.1$^-$ T cells (**Figure 3B**). Furthermore, the median fluorescent intensity (MFI) for Bcl6 expression was significantly higher at day 8 p.i. in the NK1.1$^+$ population, and this trend continued at day 11 p.i. (**Figure 3C**). Although Th1 and Tfh cells are the predominant CD4$^+$ T helper cell subsets that emerge after *Plasmodium* infection (19), we also wanted to assess transcription factors associated with other T helper cell subsets, such as RORγt (Th17), Gata3 (Th2), and Foxp3 (Treg). No distinct populations of RORγt or Gata3-expressing CD4$^+$ T cells were seen at day 8 p.i. within either the NK1.1$^+$ or NK1.1$^-$ populations. Though there was a sizable population of Foxp3$^+$ regulatory T cells within the NK1.1$^-$ pool this was not the case for the NK1.1-expressing population (**Figure 3A** and **Supplemental Figure 4**).

CD4$^+$ T cells produce many cytokines that are essential for their effector response after *Plasmodium* infection, including

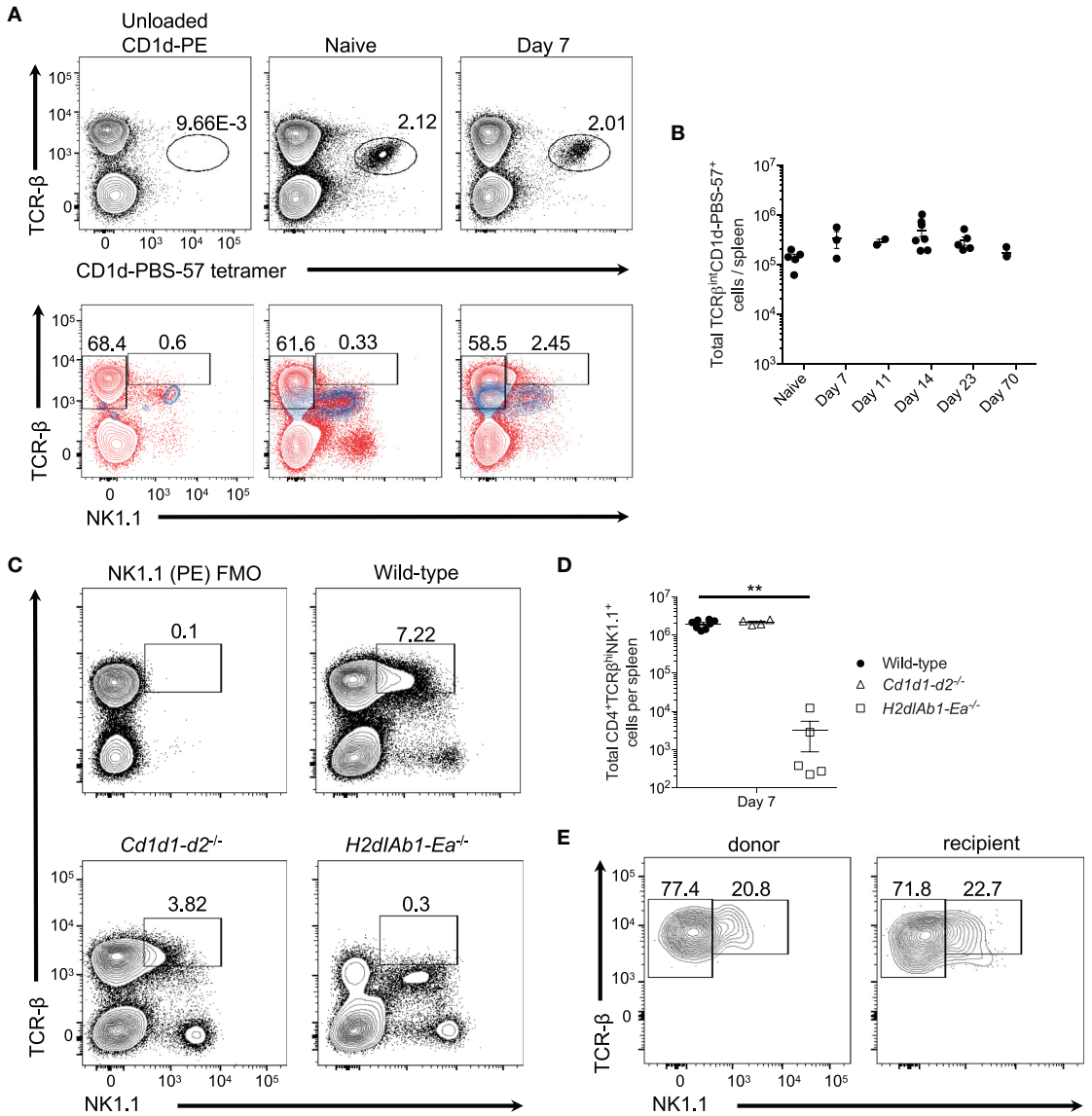

FIGURE 2 | Type I invariant NK-T cells do not expand during *P. yoelii* infection. **(A)** Top: Representative TCRβ and PBS-57 tetramer staining in naive and day 7 post-*P. yoelii* infected mice. Unloaded CD1d tetramer, negative staining control. Bottom: Representative TCRβ and NK1.1 expression (blue contour plots) on unloaded CD1d tetramer$^+$ cells (left), or CD1d-PBS-57 tetramer$^+$ cells from naïve (middle) and day 7 infected mice (right). Red contour plots represent TCRβ and NK1.1 expression on total splenocytes. Cells previously gated on live, dump- splenocytes. **(B)** Total number of live TCRβ^{int}CD1d-PBS57$^+$ type I NK-T cells per spleen. Data are representative of two independent experiments (error bars, s.e.m.). **(C)** Representative TCRβ and NK1.1 expression at day 7 post-*P. yoelii* infection in wild-type, CD1d deficient (*Cd1d1-d2*$^{-/-}$), or MHC-II deficient (*H2dlAb1-Ea*$^{-/-}$) mice. FMO, fluorescence minus one staining control. **(D)** Total number of live CD4$^+$TCRβ$^+$NK1.1$^+$ T cells per spleen at day 7 post-infection. Significance calculated by one-way ANOVA Kruskal-Wallis test with *post hoc* Dunn's multiple comparisons test. $^{**}p < 0.01$. **(E)** Representative TCRβ and NK1.1 expression on endogenous CD45.1$^+$ or donor CD45.2$^+$ CD4$^+$ T cells at day 8 post-infection. Naïve (CD44^{lo}CD62L^{hi}) CD4$^+$ T cells from C57BL/6 mice (CD45.2$^+$) were sorted and transferred i.v. into congenic CD45.1$^+$ mice 1 day before challenge with *P. yoelii*. Data are representative of two independent experiments (error bars, s.e.m.).

IFN-γ, IL-21, and TNF-α (20–23). To determine if NK1.1$^+$ T cells are capable of producing these effector cytokines splenocytes were re-stimulated directly ex vivo. Interestingly, a significantly higher percentage of NK1.1$^+$CD4$^+$ T cells was found to produce IFN-γ and IL-21, but less TNF-α than NK1.1$^-$CD4$^+$ T cells. Also, a higher frequency of NK1.1$^+$CD4$^+$ T cells was found to produce two (IFN-γ$^+$IL-21$^+$, IFN-γ$^+$TNF-α$^+$, IL-21$^+$TNF-α$^+$) or all three (IFN-γ$^+$IL-21$^+$TNF-α$^+$) cytokines than NK1.1$^-$CD4$^+$ T cells (**Figures 3D,E**). These data suggest NK1.1$^+$CD4$^+$ T cells share similarities with activated NK1.1$^-$CD4$^+$ T cells. Also, the finding that a significant proportion of NK1.1$^+$CD4$^+$ T cells produce pro-inflammatory cytokines fits with their pronounced

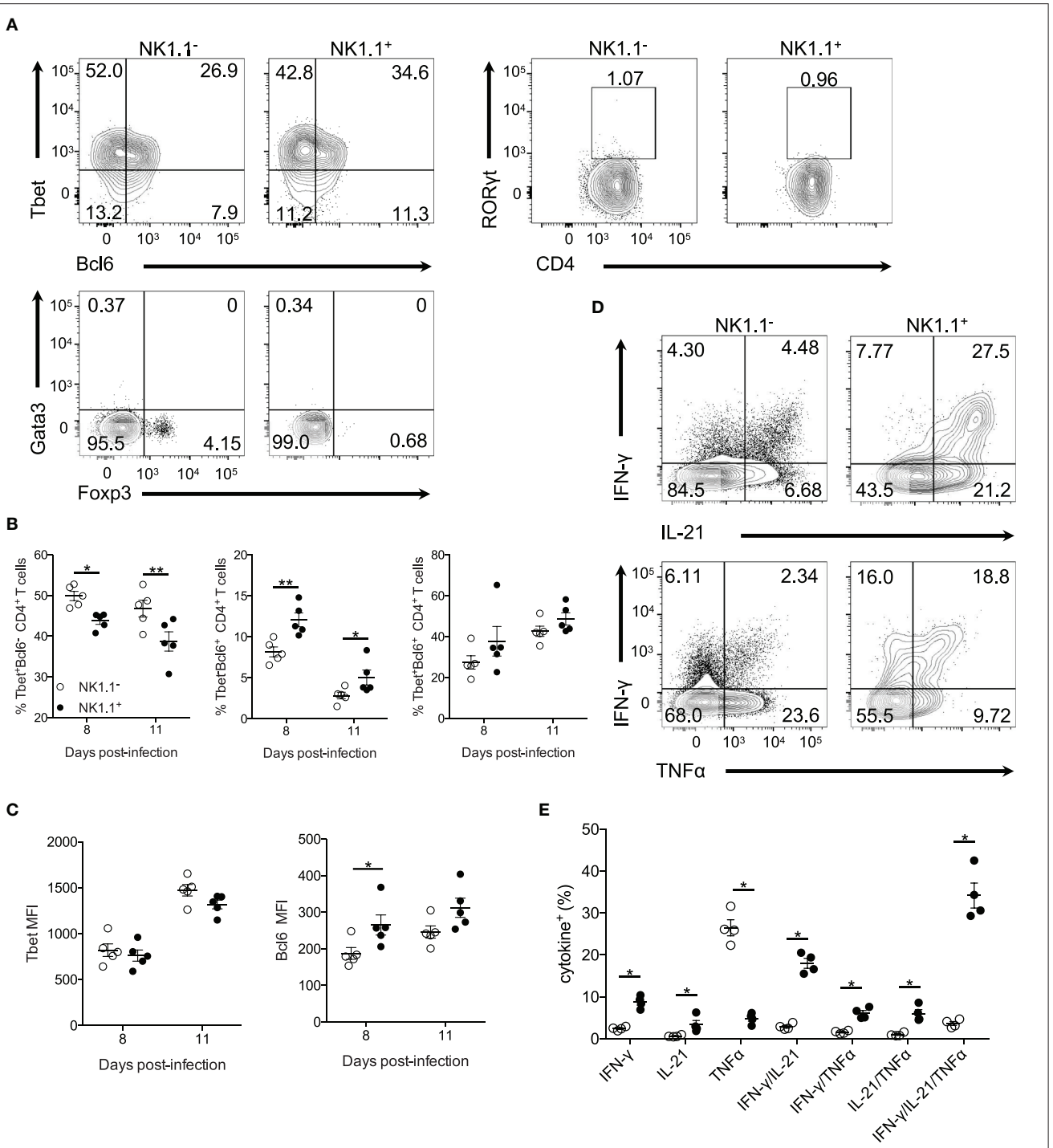

FIGURE 3 | NK1.1$^+$CD4$^+$ T cells express Tbet and Bcl6 and produce effector cytokines. **(A)** Representative intracellular staining of NK1.1$^-$ and NK1.1$^+$ CD4$^+$ T cells for the transcription factors Tbet, Bcl6, RORγt, Gata3, and Foxp3. Cells previously gated on live, CD44^{hi}CD62L^{lo}CD4$^+$TCRβ$^+$ splenocytes. Gates based on FMO controls shown in **Supplemental Figure 4**. **(B)** Frequency of NK1.1$^+$ and NK1.1$^-$ CD4$^+$ T cells expressing Tbet, Bcl6 or, Tbet and Bcl6 on days 8 and 11 p.i. An aligned rank transformation was performed on non-parametric data before determining significance by two-way ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. *$p < 0.05$, **$p < 0.01$. **(C)** Median fluorescence intensity (MFI) for Tbet and Bcl6 in NK1.1$^+$ and NK1.1$^-$ CD4$^+$ T cells on day 8 and 11 p.i. An aligned rank transformation was performed on non-parametric data before determining significance by two-way ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. *$p < 0.05$. **(D)** Representative intracellular cytokine staining of NK1.1$^-$ and NK1.1$^+$ CD4$^+$ T cells previously gated on live CD4$^+$TCRβ$^+$ T cells, on day 7 post-infection. Splenocytes were stimulated for 4 h with PMA and ionomycin in the presence of brefeldin A before being stained for IFN-γ, IL-21, and TNFα. **(E)** The frequency of single, double, or triple cytokine-producing cells at day 7 post-infection. Significance assessed via an unpaired non-parametric Mann-Whitney test. *$p < 0.05$. Data are representative of three independent experiments (error bars, s.e.m.).

effector phenotype (**Figure 1**). Together, these data indicate the expression of NK1.1 does not prevent or limit differentiation of these $CD4^+$ T cells into a particular T helper subset, as $NK1.1^+CD4^+$ T cells display characteristics of Th1 and Tfh cells. Although, it appears these cells slightly favor Tfh over Th1 cell differentiation compared to $NK1.1^-$ T cells.

$NK1.1^+CD4^+$ T Cells Are Predominantly Tfh-Like Cells

The capacity to produce IL-21, a cytokine involved in germinal center B cell reactions and the high expression of Bcl6 by $NK1.1^+CD4^+$ T cells suggested that these cells might represent an early Tfh cell population. Therefore, we evaluated additional markers associated with Tfh cell differentiation on activated $CD4^+$ T cells ($CD44^{hi}CD62L^{lo}$). ICOS, a co-stimulatory molecule implicated in the induction and maintenance of Tfh cells (24–26), was expressed on both NK1.1 positive and negative $CD4^+$ T cells on day 5 after infection. (**Figures 4A,B**). By day 8 p.i. greater than 90% of the $NK1.1^+CD4^+$ T cells expressed ICOS, and the frequency of $ICOS^+$ cells remained significantly higher amongst the $NK1.1^+CD4^+$ T cells through the peak of infection (**Figures 4A,B**). Despite a higher rate of ICOS expression amongst $NK1.1^+CD4^+$ T cells, there were more $ICOS^+$ $NK1.1^-CD4^+$ T cells on day 5 p.i. By day 8 both populations of $CD4^+$ T cells showed a significant expansion in $ICOS^+$ cells that continued through day 11 with both populations showing a substantial reduction in ICOS expression at day 14 p.i. However, the gap in the number of $ICOS^+$ cells seen on day 5 p.i. closed by day 8 p.i., as a similar number of $ICOS^+$ cells was seen within the $NK1.1^-$ and $NK1.1^+$ populations and numbers remained comparable through day 14 (**Figure 4C**).

As ICOS signaling is implicated in Tfh cell priming, expression of PD-1 and CXCR5—lineage-defining Tfh cell proteins—was assessed. At day 5 p.i. a significant proportion of $NK1.1^+CD4^+$ T cells already expressed PD-1 and CXCR5 compared to $NK1.1^-CD4^+$ T cells. Although the expression of PD-1 and CXCR5 significantly increased amongst both NK1.1 positive and negative $CD4^+$ T cells between days 5 and 8 p.i., $NK1.1^+CD4^+$ T cells more frequently expressed PD-1 and CXCR5 relative to $NK1.1^-CD4^+$ T cells throughout the first two weeks of infection (**Figures 4D,E**). Also, a higher proportion of $NK1.1^+CD4^+$ T cells expressed CXCR5 and PD-1 after *P. chabaudi* infection than conventional $CD4^+$ T cells (**Supplemental Figure 1**). Moreover, superimposed plots of PD-1 and CXCR5 expression indicated that $NK1.1^+$ T cells expressed significantly more PD-1 and CXCR5 on a cell to cell basis relative to $NK1.1^-$ T cells, as shown by MFI at day 8 p.i. (**Figures 4F,G**). In support of the data in **Figure 3**, Bcl6 expression was detected in both NK1.1 positive and negative $PD\text{-}1^+CXCR5^+$ cells, but the expression of Bcl6 was significantly higher in $NK1.1^+$ T cells (**Figure 4H**). Hence, the expression of ICOS, PD-1, CXCR5 and Bcl6 by $NK1.1^+$ and $NK1.1^-$ $CD4^+$ T cells defines these cells as Tfh-like cells. Ultimately, the total number of $NK1.1^+$ and $NK1.1^-$ Tfh-like cells did not differ dramatically over the course of *P. yoelii* infection, although both populations showed a significant expansion in cell numbers from day 5–8 and day 8–11 p.i., and they showed a significant reduction in numbers from day 11–14 p.i. (**Figure 4I**). Together, these data indicate $NK1.1^+CD4^+$ T cells are primed to preferentially adopt a Tfh cell phenotype, although a proportion of the cells are capable of expressing features associated with Th1 cells (Tbet and IFN-γ), suggesting that $CD4^+$ T cells, in general, are not fully committed to a Tfh cell fate at this time after infection. Furthermore, an increase in the expression of PD-1 and CXCR5 suggests that the Tfh-like $NK1.1^+$ cells have a distinct, unique identity within the total Tfh cell pool.

$NK1.1^+$ Tfh-like Cells Interact With Plasmablasts

Our observations thus far indicate $NK1.1^+CD4^+$ T cells expand rapidly following acute *P. yoelii* infection and favor the adoption of an $ICOS^+PD\text{-}1^{high}CXCR5^{high}$ Tfh-like phenotype. As such, we sought to assess the nature of $NK1.1^+$ Tfh cell help. Surprisingly, an in-depth ex vivo analysis revealed a substantial number of $NK1.1^+$ Tfh-like cells complexed to or interacting with other immune cells (termed doublets, as indicated by high FSC-H and FSC-A) at day 8 p.i. Indeed, $NK1.1^+$ Tfh-like cells were more likely to be found as doublets when compared to $NK1.1^-$ Tfh-like cells (**Figures 5A,B**). Conversely, the total number of singlet $NK1.1^-$ Tfh-like cells was significantly greater than doublet $NK1.1^-$ Tfh-like cells, suggesting $NK1.1^-$ Tfh-like cells less frequently formed stable interactions with other immune cells (**Figure 5B**).

We, therefore, hypothesized $NK1.1^+$ Tfh-like cells promoted the T-cell dependent wave of plasmablast differentiation that is characteristic of an acute *P. yoelii* infection. Conjugate formation was assessed during early *P. yoelii* infection to determine if plasmablasts ($B220^{low}CD138^+$) interact with $NK1.1^+$ Tfh-like cells. At day 8 p.i., a higher percentage and number of $NK1.1^+$ Tfh-like cells were found complexed to plasmablasts than $NK1.1^-$ Tfh-like cells (**Figures 5C,D**). In contrast, a significantly higher frequency of $NK1.1^-$ Tfh-like cells was found complexed to $B220^+$ B cells, although no significant difference in the number of $NK1.1^-$ and $NK1.1^+$ Tfh-like cells interacting with B cells was observed (**Figure 5E**). Also, the majority of the plasmablasts interacting with $NK1.1^+$ Tfh-like cells were class-switched, while $NK1.1^-$ Tfh-like cells interacted equally with IgM^+ and IgM^- plasmablasts (**Figure 5F**). As a whole, these data indicate that while a similar number of $NK1.1^-$ Tfh-like cells and $NK1.1^+$ Tfh-like cells interact with B cells, $NK1.1^+$ Tfh-like cells are more likely to be found complexed with plasmablasts.

Further examination of plasmablasts at day 8 p.i. revealed that these cells uniformly express PD-L1 (**Figure 6A**). As $NK1.1^+$ $CD4^+$ T cells express high amounts of the PD-L1 receptor PD-1, we hypothesized these cells might be more prone to undergo apoptosis than $NK1.1^-$ $CD4^+$ T cells. In support of this argument, previous findings have linked PD-1 signaling to the regulation of anti-apoptotic protein expression (27–29). Propidium iodide uptake, and Annexin V expression were evaluated by flow cytometry to determine if a higher frequency of $NK1.1^+$ $CD4^+$ T cells are undergoing apoptosis compared to $NK1.1^-$ $CD4^+$ T cells. Indeed $NK1.1^+CD4^+$ T

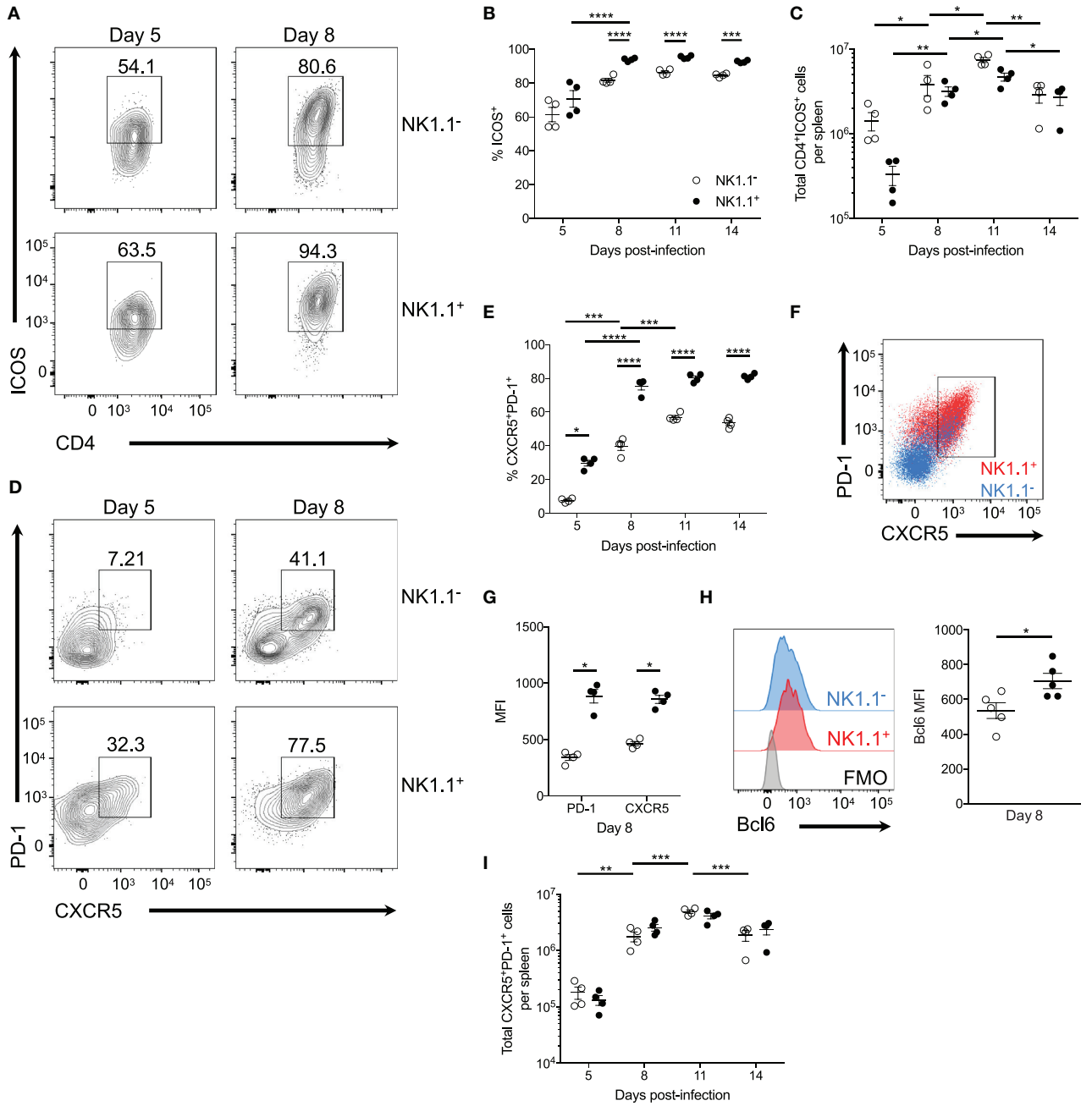

FIGURE 4 | NK1.1$^+$CD4$^+$ T cells are primarily Tfh-like cells. **(A)** Representative ICOS expression among live activated (CD44^{hi}CD62L^{lo}) NK1.1 positive and negative CD4$^+$TCRβ$^+$ T cells at days 5 and 8 post-infection. **(B)** Frequency of ICOS expression among live activated NK1.1 negative or positive CD4$^+$ T cells between days 5 and 14 post-infection. **(C)** Total number of live activated NK1.1$^-$ or NK1.1$^+$ ICOS$^+$CD4$^+$TCRβ$^+$ T cells per spleen. **(D)** Representative PD-1 and CXCR5 expression on live activated NK1.1 negative or positive CD4$^+$ T cells at days 5 and 8 post-infection. **(E)** Frequency PD-1$^+$CXCR5$^+$ of live activated CD4$^+$TCRβ$^+$ T cells. **(F)** PD-1 and CXCR5 expression overlay of NK1.1$^+$ (red) or NK1.1$^-$ (blue) CD4$^+$TCRβ$^+$ T cells at day 8 post-infection. **(G)** MFI of PD-1 and CXCR5 expression. **(H)** Intracellular Bcl6 staining of live activated NK1.1$^+$ or NK1.1$^-$ CXCR5$^+$PD-1$^+$ cells at day 8 post-infection and MFI of Bcl6 expression. **(I)** Total number of live activated NK1.1 positive or negative CXCR5$^+$PD-1$^+$ cells at day 8 post-infection. MFI, median fluorescence intensity. **(B,C,E,I)** An aligned rank transformation was performed on non-parametric data before determining significance by two-way ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. **(G,H)** Significance assessed via an unpaired non-parametric Mann-Whitney test. *$p < 0.05$. Data are representative of three independent experiments (error bars, s.e.m.).

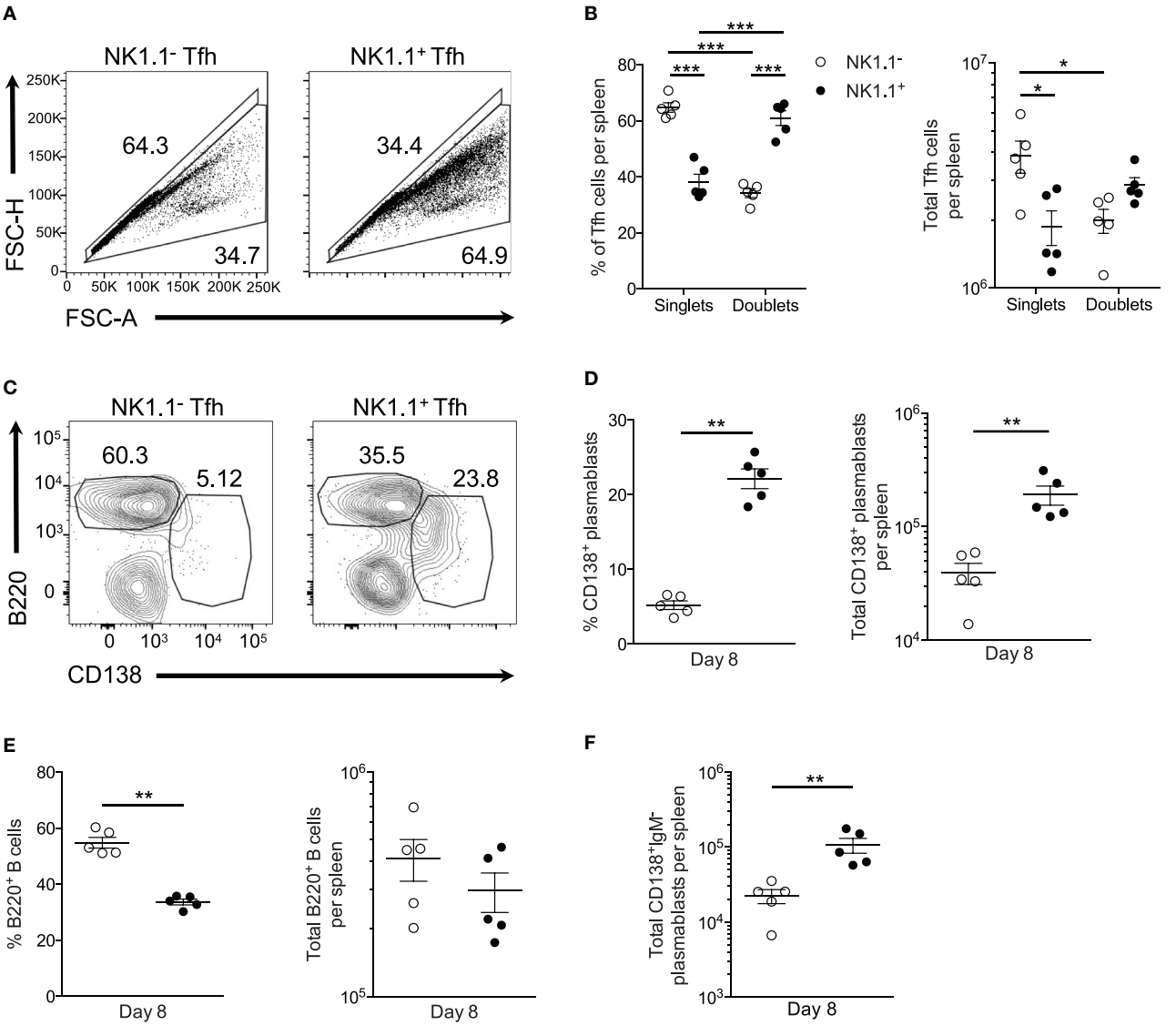

FIGURE 5 | NK1.1$^+$ Tfh-like cells frequently complex with plasmablasts. **(A)** Gating strategy for doublet (FSC-A^{high}FSC-H^{high}) and singlet (FSC-A^{low}FSC-H^{low}) live NK1.1 positive or negative Tfh-like (CXCR5$^+$PD-1$^+$) cells at day 8 post-infection. **(B)** Frequency and the total number of live singlet and doublet NK1.1 positive or negative Tfh-like cells per spleen at day 8 post-infection. An aligned rank transformation was performed on non-parametric data before determining significance by two-way ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. *$p < 0.05$, ***$p < 0.001$. **(C)** Representative B220 and CD138 staining on live doublet NK1.1 negative or positive Tfh-like cells at day 8 post-infection. **(D)** Frequency and the total number of live CD138$^+$B220^{lo} plasmablasts interacting with NK1.1 negative or positive Tfh-like cells per spleen. **(E)** Frequency and the total number of live B220$^+$ B cells interacting with NK1.1 negative or positive Tfh-like cells per spleen. **(F)** Total number of live class-switched IgM$^-$CD138$^+$ plasmablasts interacting with NK1.1 negative or positive Tfh-like cells per spleen. **(D–F)** Significance calculated by an unpaired nonparametric Mann-Whitney test. **$p < 0.01$. Data are representative of three independent experiments (error bars, s.e.m.).

cells more frequently expressed the early apoptosis indicator Annexin V from day 5 to day 14 after infection relative to NK1.1$^-$CD4$^+$ T cells (**Figures 6B,C**). Furthermore, significantly more Annexin V$^+$ NK1.1$^+$CD4$^+$ T cells were identified in the spleen at days 8, 11, and 14 relative to NK1.1$^-$CD4$^+$ T cells (**Figure 6D**). There was also an increase in the frequency and number of AnnexinV$^+$PI$^+$ NK1.1$^+$CD4$^+$ T cells compared with NK1.1$^-$CD4$^+$ T cells from day 5–11 p.i., while a higher percentage of NK1.1$^-$CD4$^+$ T cells were AnnexinV$^+$PI$^+$ at day 14 p.i. (**Figures 6B,E,F**). Although, the difference in total AnnexinV$^+$PI$^+$ cells at day 14 was not significantly different between the two populations (**Figure 6F**). Together, this data suggests that NK1.1$^+$CD4$^+$ T cells more readily undergo apoptosis relative to NK1.1$^-$CD4$^+$ T cells and offers one explanation as to why NK1.1$^+$ T cells do not persist following resolution of acute infection.

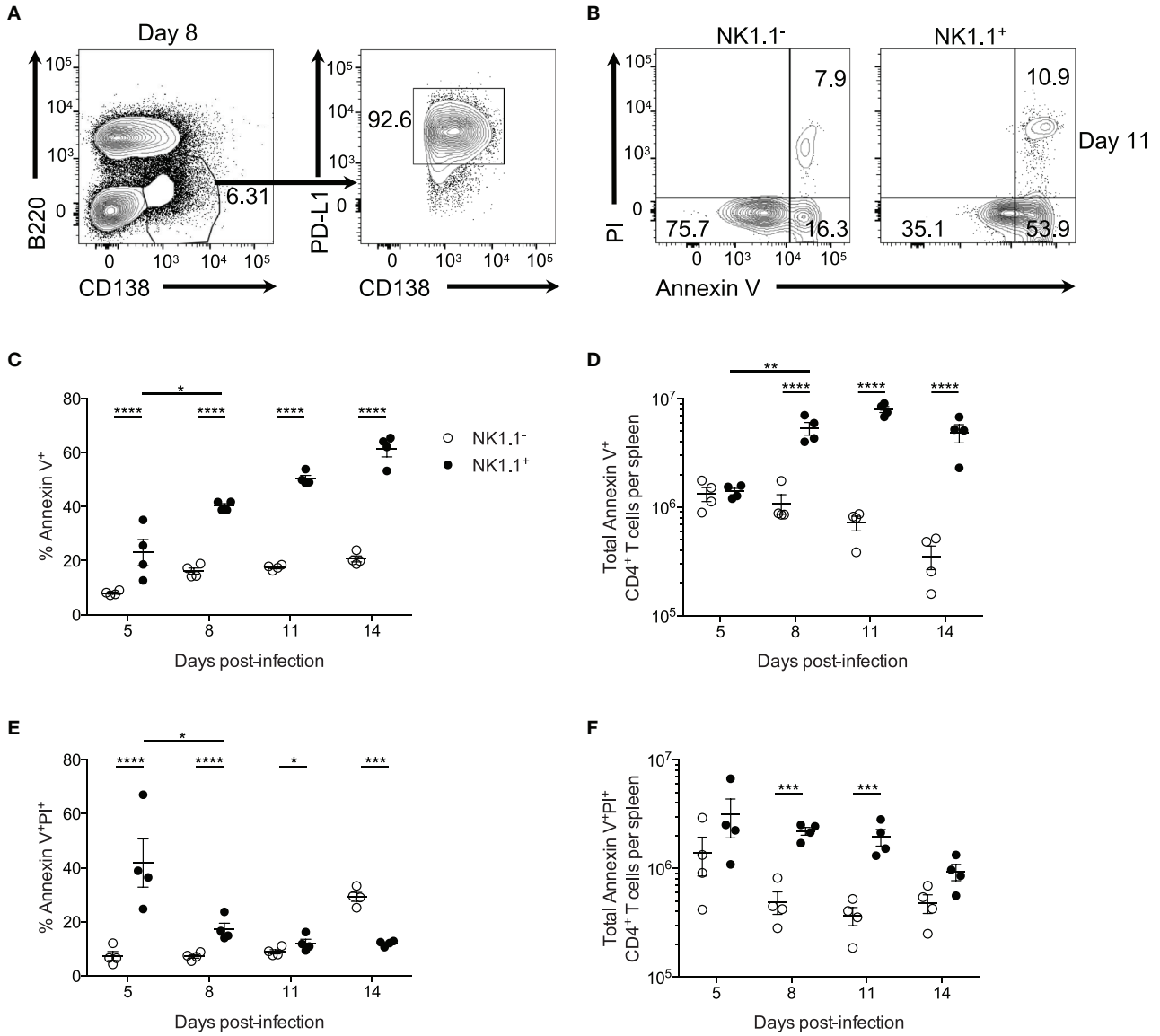

FIGURE 6 | NK1.1$^+$CD4$^+$ T cells undergo accelerated apoptosis. **(A)** Representative B220 and CD138 expression at day 8 post-infection. PD-L1 and CD138 expression of gated area at left. **(B)** Typical PI and Annexin V staining of NK1.1 negative or positive CD4$^+$TCRβ$^+$ T cells at day 11 post-infection. **(C)** Frequency and **(D)** the total number of Annexin V$^+$ NK1.1 negative or positive CD4$^+$ T cells per spleen. **(E)** Frequency and **(F)** total number of Annexin V$^+$PI$^+$ NK1.1 negative or positive CD4$^+$ T cells per spleen. An aligned rank transformation was performed on non-parametric data before determining significance by two-way ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data are representative of two independent experiments (error bars, s.e.m.).

NK1.1$^+$CD4$^+$ T Cells Promote Early Plasmablast Formation

To assess the contribution of NK1.1$^+$ T cells to early plasmablast differentiation, we sought to deplete this population during *P. yoelii* infection by utilizing an anti-NK1.1 depleting Ab. Treatment with anti-NK1.1 efficiently depleted NK1.1-expressing cells in most cases (**Figure 7A**), resulting in significantly fewer splenic TCRβ^{hi}NK1.1$^+$ T cells at day 7 p.i. (**Figure 7B**). No impact on total NK1.1$^-$CD4$^+$ T cell numbers was observed during administration of the anti-NK1.1 Ab. As anticipated, the depletion of NK1.1$^+$CD4$^+$ T cells, resulted in significantly fewer NK1.1$^+$ Tfh-like cells at day 7 p.i. (**Figure 7C**). A decline in NK1.1$^-$ Tfh-like cells was also observed at this time with anti-NK1.1 treatment.

Plasmablast differentiation was assessed at day 7 p.i. to determine if NK1.1$^+$ T cells participate in early Ab production. Depletion of NK1.1-expressing cells resulted in a significant decrease in the frequency and the total number of plasmablasts at day 7 p.i. relative to isotype control Ab-treated mice (**Figures 7D,E**). Furthermore, substantially fewer class-switched

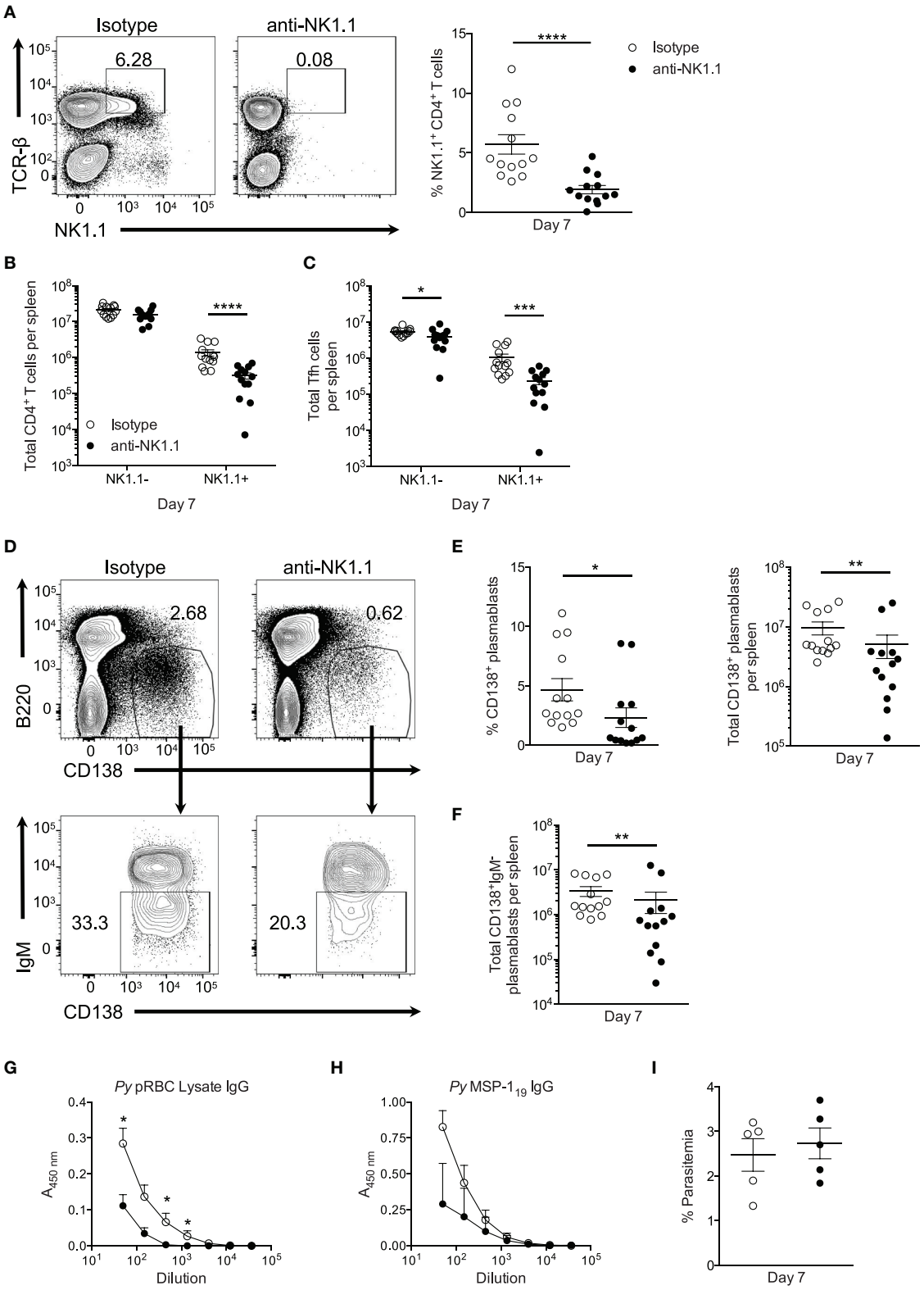

FIGURE 7 | Depletion of NK1.1$^+$CD4$^+$ T cells inhibits parasite-specific Ab production. **(A)** The frequency of NK1.1 staining of live TCR-β^{hi}NK1.1$^+$ CD4$^+$ T cells in isotype control or anti-NK1.1 Ab-treated mice at day 7 post-infection. **(B)** Total number of live splenic NK1.1 positive or negative CD4$^+$TCRβ^{hi} T cells in isotype

(Continued)

FIGURE 7 | control or anti-NK1.treated mice at day 7 post-infection. **(C)** Total number of live splenic NK1.1 positive or negative Tfh-like (CXCR5$^+$PD-1$^+$) cells in isotype control or anti-NK1.1 Ab-treated mice. **(D)** Representative B220 and CD138 expression in isotype control or anti-NK1.1 Ab-treated mice at day 7 post-infection (above). Typical IgM staining of the gated area in **(D)** (below). **(E)** Frequency and the total number of live B220^{low}CD138$^+$ plasmablasts or **(F)** IgM$^-$ class-switched plasmablasts in isotype control or anti-NK1.1 Ab-treated mice. **(A–C,E,F)** Significance determined via an unpaired nonparametric Mann-Whitney test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. **(G)** Relative pRBC-lysate specific or **(H)** MSP-1_{19}-specific IgG Ab titer at day 7 post-infection in isotype control or anti-NK1.1 Ab-treated mice. Significance determined by two-way ANOVA *post hoc* Bonferroni's multiple comparisons test. $^{**}p < 0.01$, $^{****}p < 0.0001$. **(I)** Peripheral blood parasite load in isotype control or anti-NK1.1 Ab-treated mice. Data are representative of three combined experiments **(A–F)** or three independent experiments **(G–I)** (error bars, s.e.m.).

IgM$^-$ plasmablasts were observed in mice treated with anti-NK1.1 (**Figures 7D,F**). Assessment of serum Ab production revealed a reduction in pRBC lysate and MSP-1_{19}—specific IgG Abs in NK1.1-depleted mice (**Figures 7G,H**). Depletion of NK1.1$^+$ cells, which includes innate NK cells–a potential source of early IFN-γ-did not significantly impact parasitemia, as no measurable difference in parasite burden occurred before day 7 p.i. (**Figure 7I**). However, we cannot rule out that depletion of innate NK cells contributed to the observed reduction in plasmablast and Ab production with Ab treatment. We can conclude that the effects of anti-NK1.1 treatment on plasmablast and parasite-specific Ab production were not due to depletion of CD1d-dependent NK-T cells, as no difference in the output of MSP-1_{19}-specific IgG between WT and *cd1d-d2*$^{-/-}$ mice was observed (**Supplemental Figure 2B**). Our attempt to assess if the absence of NK1.1$^+$ cells impacts parasite burden and Ab production beyond day 7 p.i. were unsuccessful, as continuous treatment with anti-NK1.1 Abs or a change in the treatment regimen to delivering the Abs every 3 days did not efficiently deplete NK1.1$^+$CD4$^+$ T cells long-term (*data not shown*). As a whole, these data indicate that NK1.1-expressing CD4$^+$ T cells interact with B cells and plasmablasts after *P. yoelii* infection, and suggest that they help to promote early plasmablast expansion, class-switching, and Ab production, which may or may not be due to direct interactions between these cell types.

DISCUSSION

The precise role of CD1d-restricted NK-T cells during blood-stage *Plasmodium* infection remains controversial. Previous literature, for instance, indicates that *P. yoelii*-infected CD1d-deficient mice exhibit delayed parasite clearance (30) and harbor greater parasite burden (author's unpublished observation) relative to WT mice. Although CD1d-deficient mice mount a moderately inadequate class-switched Ab response during *P. berghei* ANKA infection (9), and others (10) have observed no significant defect in parasite-specific Ab production during blood-stage *P. yoelii* infection. Here we show that CD1d deficient mice display higher peak parasitemia but have no defect in parasite-specific Ab production (**Supplemental Figure 2**) or evidence of CD1d-restricted T cells assuming a Tfh cell phenotype. Furthermore, this study demonstrates infection-induced NK1.1$^+$ CD4$^+$ T cells are critically reliant on MHC-II antigen presentation for their development.

As a whole, NK1.1$^+$CD4$^+$ T cells appear to represent a CD1d-independent source of highly functional early Tfh cells. However, the phenomena of NKT-like MHC-restricted T cells are not exclusively restricted to *Plasmodium* infection. In fact, >90% of virus-specific MHC-I- and MHC-II-restricted CD8$^+$ and CD4$^+$ T cells express markers associated with NK cells including NK1.1 (31). Furthermore, CD1d-independent CD4$^+$ and CD8$^+$ NKT-like cells identified in humans preferentially expand with age (32). Similarly, NK1.1-expressing CD8$^+$ CD1d-independent T cells develop in mice following LPS-primed DC immunization. In this case, NK-like CD8$^+$ T cells expressed diverse TCRαβ chains and suppressed Ag-specific T cell responses (14). As the NK1.1$^+$CD4$^+$ T cells identified in this study are not CD1d-restricted, these cells are most likely derived from a pool of diverse TCRαβ chains.

How then are MHC-II-restricted NK1.1$^+$CD4$^+$ T cells categorized amongst conventional CD4$^+$ T cells and NK-T cells? Our findings indicate that NK1.1$^+$ and NK1.1$^-$ CD4$^+$ T cells are composed of a heterogeneous population of cells expressing markers associated with Th1 and Tfh cells, similar to what has been reported by others (21, 23, 33–35), further supporting the idea that T cells transition through an intermediate state prior to full commitment toward Th1 or Tfh cell differentiation (35). Perhaps, NK1.1 expression defines one of these intermediate states. The distinguishing feature of NK1.1$^+$ CD4$^+$ T cells is their heightened expression of ICOS, CXCR5, PD-1, and Bcl6, suggesting they favor a Tfh cell fate. However, examination of CD4$^+$ T cells at a time (Day 24) when the germinal response is prevalent revealed that the GC Tfh cells (PD-1^{hi}CXCR5$^+$) consist of only NK1.1$^-$ CD4$^+$ T cells (*data not shown*). Indeed, we observed a significant contraction in the number of NK1.1-expressing CD4$^+$ T cells at this time due to an increased rate of programmed cell death (**Figures 1**, **6**). Rather than favoring their entry into the B cell follicle the early and heightened expression of proteins associated with a Tfh cell fate may instead favor interactions with B cells serving the purpose to promote and support early plasmablast differentiation.

Another factor that could influences CD4$^+$ T cell effector fate determination is the length of peptide:TCR interaction (dwell time), as well as antigen dose (36, 37). As Th1 and Tfh cell differentiation is finely balanced by the ratio of Blimp-1:Bcl6, it is possible that high amounts of peptide:MHC expression by DCs during the initial DC:CD4$^+$ T cell interactions primarily lead to induction of ICOS and subsequent Bcl6 expression, as well as upregulation of NK1.1. This outcome could favor the early formation of Tfh-like cells, which appear to be responsible for early T-dependent plasmablast differentiation—a key event in the control of *P. yoelii* infection. Indeed, depletion

of NK1.1-expressing cells yields a dramatic defect in initial parasite-specific plasmablast production. However, as most plasmablasts are NK1.1$^+$ doublets, it is likely NK1.1$^+$ T cell-complexed plasmablasts are simultaneously depleted. Strong NK1.1$^+$ Tfh:plasmablast interaction suggests that these NK1.1$^+$ Tfh-like cells are essential for plasmablast differentiation or immediate survival. However, the extrafollicular plasmablast response is short-lived, and these non-mutated Ab-secreting cells do not persist in the spleen for an extended period, implying the majority of NK1.1$^+$ Tfh-like cells are short-lived effectors. As such, the NK1.1$^-$ T cells (which less frequently interacted with plasmablasts) that express Tfh cell markers may proceed into the B cell follicle to promote GC formation and continue their differentiation into GC Tfh cells.

As mentioned, NK1.1 belongs to the NKRP1 (NK receptor protein) family. Unlike killer cell Ig-like receptors (KIR), NKRP1 family members do not bind MHC class-I-like ligands. Instead, NKRP1 receptors in mice, rats, and humans have been shown to engage Clec2 (C-type lectin-like receptor 2) subfamily proteins (38). Engagement of NKRP1 receptors can lead to activation or inhibition of NK cells, although NK1.1 does not possess a consensus ITIM in its cytoplasmic domain (39), suggesting it lacks inhibitory function. NK1.1 possesses a positively charged arginine residue near its extracellular region in its transmembrane domain (40), which indicates that NK1.1 could interact with negatively charged transmembrane proteins such as the CD3 subunits on T cells to promote signaling through the TCR, which could result in enhanced activation or proliferation of T cells.

Despite these insights, the factors responsible for promoting NK1.1 expression itself remain elusive. It was previously shown that CD8$^+$ T cells upregulate NK1.1 within 48–72 h in response to IL-2, IL-4, and IL-15 addition *in vitro* (13). We found that a higher percentage of NK1.1$^+$ T cells displayed IL-2Rα and β expression upon their emergence at day 5 p.i. compared to NK1.1$^-$CD4$^+$ T cells (**Supplemental Figure 5**), suggesting that they preferentially respond to IL-2 or IL-15. While the purpose and function of NK1.1 expression by CD4 and CD8 T cells is still unclear, its appearance likely represents a state of activation rather than a distinct cell lineage.

Ultimately, defective parasite-specific Ab production failed to result in the acceleration of parasite burden. Therefore, how essential are NK1.1$^+$ T cells in the induction of protective immunity? Ineffective depletion of NK1.1$^+$ cells during late *P. yoelii* infection may partially explain why a robust protective phenotype did not emerge in this infection model. Until better tools become available for studying the function of NK1.1 on T cells, it will be difficult to understand the importance of this cell population in the immune response to *Plasmodium*. Based on the depletion studies, it is likely that this protein does not play an essential role in the immune response due to redundancy in the system. Therefore, confirmation of the localization of NK1.1$^+$CD4$^+$ T cells solely to the extrafollicular region in the red pulp in the spleen would facilitate the use of this protein as a marker for identifying and characterizing extrafollicular Tfh cells. Moreover, a greater understanding of the development of NK1.1$^+$CD4$^+$ T cells, as well as the fate of NK1.1$^+$ Tfh-like cells, may help instruct future rational anti-malarial vaccine design.

AUTHOR CONTRIBUTIONS

DW and JS designed, analyzed and interpreted the results of the study. DW, SB, and JL carried out the experiments associated with the study. DW and JS wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02277/full#supplementary-material

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The Role of BACH2 in T Cells in Experimental Malaria Caused by *Plasmodium chabaudi chabaudi* AS

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BTB and CNC Homology 1, Basic Leucine Zipper Transcription Factor 2 (BACH2) is a transcription factor best known for its role in B cell development. More recently, it has been associated with T cell functions in inflammatory diseases, and has been proposed as a master transcriptional regulator within the T cell compartment. In this study, we employed T cell-specific *Bach2*-deficient (B6.*Bach2*$^{\Delta T}$) mice to examine the role of this transcription factor in CD4$^+$ T cell functions *in vitro* and in mice infected with *Plasmodium chabaudi* AS. We found that under CD4$^+$ T cell polarizing conditions *in vitro*, Th2, and Th17 helper cell subsets were more active in the absence of *Bach2* expression. In mice infected with *P. chabaudi* AS, although the absence of *Bach2* expression by T cells had no effect on blood parasitemia or disease pathology, we found reduced expansion of CD4$^+$ T cells in B6.*Bach2*$^{\Delta T}$ mice, compared with littermate controls. Despite this reduction, we observed increased frequencies of Tbet$^+$ IFNγ^+ CD4$^+$ (Th1) cells and IL-10-producing Th1 (Tr1) cells in mice lacking *Bach2* expression by T cells. Studies in mixed bone marrow chimeric mice revealed T cell intrinsic effects of BACH2 on hematopoietic cell development, and in particular, the generation of CD4$^+$ and CD8$^+$ T cell subsets. Furthermore, T cell intrinsic BACH2 was needed for efficient expansion of CD4$^+$ T cells during experimental malaria in this immunological setting. We also examined the response of B6.*Bach2*$^{\Delta T}$ mice to a second protozoan parasitic challenge with *Leishmania donovani* and found similar effects on disease outcome and T cell responses. Together, our findings provide new insights into the role of BACH2 in CD4$^+$ T cell activation during experimental malaria, and highlight an important role for this transcription factor in the development and expansion of T cells under homeostatic conditions, as well as establishing the composition of the effector CD4$^+$ T cell compartment during infection.

Keywords: BACH2, malaria, protozoan, T cells, inflammation

INTRODUCTION

BTB and CNC Homology 1, Basic Leucine Zipper Transcription Factor 2 (BACH2) is a leucine zipper transcription factor known for its role in B cell development. It is also involved in antibody class switch recombination and somatic hypermutation (1). More recently, it has been associated with T cell function in a number of inflammatory diseases, and as such, has been described as a master transcriptional regulator within the T cell compartment (2, 3). *BACH2* dysregulation has been associated with a number of immune disorders, including tumor suppression and control of B cell lymphomas (4). However, in some cancers it was mutated or fused with other genes leading to dysregulated expression of *BACH2* itself or BACH2 fusion protein (5, 6).

BACH2 is often down-regulated in inflammatory disorders. For example, $CD4^+$ T cells from coeliac disease patients had down-regulated *BACH2* expression associated with inflammation (7). Interestingly, *IFNγ* was highly expressed in these $CD4^+$ T cells, suggesting that BACH2 may play a role in regulating *IFNγ* expression. Several genome wide association studies have also found significant associations between the presence of single nucleotide polymorphisms in *BACH2* and susceptibility to inflammatory diseases, including rheumatoid arthritis, Crohn's disease, asthma, and multiple sclerosis (8–11). In a mouse model of multiple sclerosis (experimental autoimmune encephalomyelitis; EAE), *Bach2* was down-regulated in Th17 cells and expression was negatively associated with disease severity (12). Another study showed that *Bach2* was significantly down regulated in T cells during EAE, and this correlated with increased *Foxp3* methylation and reduced *Foxp3* expression, suggesting BACH2 influences epigenetic modification of the *Foxp3* promoter region to support thymic-derived $FoxP3^+$ regulatory T (Treg) cell development and expansion (13).

Other studies have identified additional roles for BACH2 in regulating T cell homeostasis (2, 14, 15). Control of T cell numbers is critical for immune homeostasis, and dysregulation can result in immune disorders (16–18). As mentioned above, *Bach2* expression was essential for the stability and function of Treg cells, but also plays a role in the differentiation of $CD4^+$ T cells into effector lineages, such as Th1, Th2, and Th17 cells (2, 14, 15). For example, *Bach2* knockout mice developed a Th2 cell-dependent lung disease, associated with enhanced Th2 cell cytokine production and lung inflammation (15), indicating a requirement for BACH2 in controlling Th2 cell differentiation and/or tissue recruitment. BACH2 has also been shown to promote Th1 cell responses over Th2 cell responses during infection. In a mouse model of *Listeria monocytogenes* infection, loss of BACH2 enhanced Th2 cell responses while reducing Th1 cell development (14). *Prdm1* (encoding BLIMP1) expression was increased in T cells from *Bach2* knockout mice, suggesting BACH2 may suppress T cell *Prdm1* expression (14). Thus, a potential mechanism by which BACH2 impacts $CD4^+$ T cell differentiation is by suppressing *Prdm1* expression. This would normally promote Th2 cell differentiation by down-regulating Th1 and T follicular helper (Tfh) cell lineage genes, such as *Tbx21* and *Bcl6,* respectively (19). However, BACH2 also appeared to promote $CD4^+$ T cell regulatory phenotypes over other $CD4^+$ T cell subsets. This was supported by the unrestrained Th2 cell-mediated wasting disease observed in *Bach2* knockout mice, along with upregulation of Th1, Th2, and Th17 cell-associated genes, when $CD4^+$ T cells from these mice were polarized under relevant conditions (2). BACH2 can also suppress $CD8^+$ T cell function, although this was shown to be indirect, and occurred via the inhibitory actions of Treg cells (20). Thus, in autoimmune disease and *in vitro* cell culture assays, BACH2 promotes development of a regulatory $CD4^+$ T cell phenotype, while suppressing development of effector $CD4^+$ T cells through both cell intrinsic and extrinsic mechanisms. Whether this also occurs in parasitic diseases is unknown.

Intracellular protozoan parasites that cause diseases such as malaria and leishmaniasis generally require a pro-inflammatory immune response mediated by Th1 cells for control of parasite growth (21). In the case of *Plasmodium* species that cause malaria, a robust T follicular helper (Tfh) cell response is also needed to generate protective anti-parasitic antibodies (22–25). However, disease often develops because these responses are either impaired or dysregulated. Recently, $Foxp3^-$ IL-10-producing Th1 cells (type 1 regulatory; Tr1), rather than thymus-derived $FoxP3^+$ $CD4^+$ regulatory T (Treg) cells, have also been recognized to play important roles in determining the outcome of protozoan parasitic diseases, including malaria, leishmaniasis and toxoplasmosis (26–29). IL-10 production by Tr1 cells has been shown to be governed by BLIMP (30, 31), and we recently showed that *Prdm1* expression by T cells enhanced Tr1 cell development, while suppressing Th1 cell expansion (28). This was associated with enhanced parasite burden and increased morbidity in mouse models of malaria and visceral leishmaniasis (VL) (28). Because BACH2 is thought to suppress *Prdm1* expression (32), we hypothesized that BACH2 would antagonize BLIMP1 activity in these diseases, resulting in opposing outcomes to *Prdm1*-deficient mice. BACH2 has predominantly been investigated using *Bach2*-deficient mice, which have not allowed the importance of cell, tissue or temporal expression of BACH2 to be examined. Therefore, we employed T cell-specific *Bach2* knockout mice to investigate the role of BACH2 in protozoan parasitic infections.

MATERIALS AND METHODS

Infections and Quantification of Parasite Burden

One passage mouse was infected with 200 μL cryo-preserved *P. chabaudi chabaudi* AS parasitised red blood cell (pRBC) inoculum via intravenous tail injection. When passage parasitemia reached 2–4% (typically 2–4 days post inoculation), blood was harvested and prepared for inoculation of experimental mice. Briefly, passage mice were euthanized using CO_2 inhalation, blood was harvested via cardiac bleed and washed in media {5IU heparin (Pfizer, NSW, Australia), 1% (w/v) penicillin/streptomycin [Gibco (Thermo Fischer, Walther, MA, USA)], in RPMI}. The concentration of pRBC

was adjusted to 5×10^5 per mL in RPMI/PS. Experimental mice were infected with 1×10^5 iRBC via intravenous (i.v.) tail injection.

Parasitemia was monitored via flow cytometry. Briefly, one drop of blood was collected into 200 μL of media. Fifty microliter of diluted blood was incubated with 50 μL of Syto84 [5 μM, Life Technologies (Thermo Fischer)] and Hoechst33342 [10 ug/mL, Sigma (St Louis, MO, USA)] for 30 min, at room temperature, protected from light. This was then diluted out to 6 times the original volume with RPMI, and acquired on one of three BD flow cytometers (Canto II, Fortessa 4, or Fortessa 5). FlowJo software (v.8, Treestar, CA, USA) was used to quantitate parasitemias.

L. donovani (LV9; MHOM/ET/67/HU3) was maintained by passage in B6.*Rag1*$^{-/-}$ mice. Amastigotes were isolated from chronically infected passage animals. Experimental mice were infected by injection of 2×10^7 amastigotes i.v., via the lateral tail vein. Mice were culled at different time post-infection (p.i.) indicated in the text by CO_2 asphyxiation and bled via cardiac puncture. Spleens were removed and livers perfused then removed, with parasite burden determined by qPCR, as previously described (33, 34). Hepatic and splenic mononuclear populations were isolated as previously described (28, 35).

Mice

Inbred female C57BL/6 and congenic B6.CD45.1 mice, 6 weeks of age, were purchased from the Animal Resource Center (ARC; Canning Vale, WA, Australia). B6.*Cd4-Cre* transgenic mice (36) were crossed with B6.*Bach2*-floxed transgenic mice (37) to generate T cell-specific BACH2-deficient C57BL/6 (B6.*Bach2*$^{\Delta T}$) mice. Littermates lacking the *Cd4-cre* transgene (B6.*Bach2*$^{fl/fl}$) were used as controls. It should be noted that the *Bach2* gene will be depleted in both CD4$^+$ and CD8$^+$ T cells in these animal due to expression of both CD4 and CD8 on double positive thymocytes during T cell development. Female mice were used in all experiments and were age-matched, and bred and maintained in-house at QIMR Berghofer (Brisbane, Australia) under pathogen-free conditions. All animal procedures were conducted with the approval of the QIMR Animal Ethics Committee under the animal ethics number A02-634M and in accordance with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian NHMRC, Canberra).

In vitro Stimulation of CD4$^+$ T Cells

CD4$^+$ T cells were isolated from spleens using the Miltenyi mouse CD4$^+$ T cell isolation kit, according to manufacturer's guidelines (Miltenyi, Biotec, Bergisch Gladbach, Germany). CD4$^+$ T cells (4×10^5/well) were then cultured with αCD28 (1 μg/mL, clone 37.51, BioLegend, San Diego, CA) and plate bound αCD3ε mAb (wells coated with 1 μg/mL for 4 h at 37°C, 5% CO_2, clone 145-2C11, BioLegend) in a 96-well plate, for 5 days (3 days for Tr1 and any comparative conditions) at 37°C, 5% CO_2. Cells were cultured in DMEM or RPMI (Tr1 cell conditions only), both supplemented with 10% (v/v) fetal calf serum, 10 mM L-glutamine, 100 U/mL penicillin, 100 ng/mL streptomycin. Cell culture media was supplemented with Th0 (10 ng/mL IL-2), Th1 (10 ng/mL IL-2, 10 ng/mL IL-12, 10 ng/mL αIL-4), Th2 (10 ng/mL IL-2, 40 ng/mL IL-4, 10 ng/mL αIFNγ), or Th17 (20 ng/mL IL-6, 1 ng/mL TGFβ, 10 ng/mL IL-23, 10 ng/mL IL-1β, 10 ng/mL TNFα, 10 ng/mL αIL-4, 10 ng/mL αIFNγ) cytokines (eBioSciences) for CD4$^+$ T cell polarization (38). After 5 days, culture supernatants were harvested and cytokine concentrations were assessed using mouse Cytometric Bead Arrays: Inflammatory Cytokine CBA Kit, Th1/Th2/Th17 Cytokine Kit, and IFNγ and IL-10 Flex Sets (BD Biosciences, Franklin Lakes, NJ, USA), according to manufacturer's guidelines.

Generation of Mixed-BM Chimeric Mice

Mixed bone marrow chimeric mice were generated by lethally irradiating mice with two doses of 5.5 cGy and subsequently engrafting with 10^6 freshly isolated bone marrow cells i.v., via the lateral tail vein, as previously described(39). Irradiated recipients were engrafted with either a 50:50 or 30:70 mix of congenic (CD45.1) C57BL/6 and B6.*Bach2*$^{\Delta T}$ (CD45.2) bone marrow cells, as indicated in the text.

Flow Cytometry

All organ-derived mononuclear cells were prepared as previously described (28, 39). Fluorescein-conjugated mAbs against CD4 (GK1.5), CD8α (53-6.7), TCRβ (H57-597), CD11a (M1714), CD49d (R1-2), Tbet (4B10), IFNγ (XMG1.2), IL-10 (JES5-16E3), CD45.1 (A20), and CD45.2 (104) (Biolegend) were used. Dead cells were excluded from analysis using LIVE/DEAD Fixable Aqua Stain (Invitrogen), as per manufacturer's instructions. Both cell surface and intracellular staining was undertaken according to methods previously described (28), with all samples acquired on a BD LSRFortessa (BD Biosciences). Gating strategies used for analysis are outlined in **Figures 3**, **6**. For analysis of intracellular IFNγ and IL-10, cells were stimulated for 3 h at 37°C and 5% CO_2 in the presence of PMA (Sigma) and Ionomycin (Sigma) in addition to Brefeldin A (Sigma), as previously described (28).

Statistical Analysis

Statistical analysis was performed exclusively in GraphPad Prism 5 and 6 (GraphPad Software, La Jolla, CA). A non-parametric, un-paired Mann-Whitney test was used for comparisons between two groups. A p-value of < 0.05 was considered significant. Graphs depict mean ± SEM.

RESULTS

T Cell-Specific BACH2 Influences Th2 and Th17 Differentiation

To study the role of BACH2 in CD4$^+$ T cells, we generated T cell-specific *Bach2* knockout mice by crossing *Cd4-cre* transgenic mice with *Bach2* floxed (B6.*Bach2*$^{\Delta T}$) mice. Littermates lacking the *Cd4-cre* transgene (B6.*Bach2*$^{fl/fl}$) were used as controls. CD4$^+$ T cells were isolated from spleens of naïve B6.*Bach2*$^{fl/fl}$ and B6.*Bach2*$^{\Delta T}$ mice and cultured with αCD3 and αCD28 mAbs in the presence of neutral (Th0), Th1, Th2, or Th17 cell polarizing cytokines. CD4$^+$ T cell *Bach2*-deficiency resulted in multiple changes in cytokine production (**Figure 1A**), consistent with

previous findings (2, 14, 15). These changes included increased TNF, IL-10, and IL-13 production under all conditions tested. In addition, B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells produced significantly more IL-4 under Th0 and Th2 cell conditions, and significantly increased levels of IL-17A were measured under Th17 cell conditions. A notable exception to BACH2-mediated changes in CD4$^+$ T cell cytokine production was IFNγ, whereby no consistent, BACH2-dependent change was observed under any cell culture condition tested. This was consistent with no change in the generation of Tbet$^+$ B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells, compared with controls, under Th1 cell conditions (**Figure 1B**). However, a decrease in the generation of Tbet$^+$ B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells was found under Th0 cell conditions, relative to control cells (**Figure 1B**). A similar observation was made for GATA3$^+$ B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells cultured under Th2 cell culture conditions, despite corresponding increased IL-4 and IL-13 production (**Figure 1B**). Again, a decrease in the generation of GATA3$^+$ B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells was found under Th0 cell conditions (**Figure 1B**). BACH2 suppressed expansion of RORγ$^+$ CD4$^+$ T (Th17) cells under Th17 polarizing conditions, consistent with increased levels of IL-17A in the absence of BACH2 (**Figure 1B**). We also observed a significant increase in IL-10 levels in all culture conditions with B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells, compared with controls (**Figure 1A**). Significantly, under Th1 cell conditions, Tbet expression was maintained in B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells (**Figure 1B**) and IL-10 production was increased (**Figure 1A**), compared with control cells, indicating BACH2 suppressed Tr1 cell development. Given that BLIMP1 is critical for CD4$^+$ T cell IL-10 production (28), this finding suggests that BACH2 may inhibit CD4$^+$ T cell BLIMP1-dependent IL-10 production.

The Role of BACH2 in Experimental Malaria

The outcome of *Plasmodium* infection depends on host CD4$^+$ T cell responses (40–43), and BLIMP1-dependent Tr1 cell responses have a major influence on disease outcome (28). Since BACH2 influenced effector CD4$^+$ T cell subset development *in vitro*, and in particular the development of Tr1 cells, we hypothesized that BACH2 would influence cellular responses during infection, and consequently, affect disease outcome. B6.*Bach2*$^{\Delta T}$ and control mice were infected with *Plasmodium chabaudi chabaudi* AS (*P. chabaudi*) that causes an acute, resolving infection in C57BL/6J mice (44). We chose this malaria model because there is a clear requirement for anti-parasitic Th1 cell responses to control parasite growth and Tr1 cell responses to control associated inflammation and restrict tissue pathology (45–47). We found no effect of BACH2 deficiency on control of parasite growth in this model (**Figure 2A**), as well as no change in body weight (**Figure 2B**), an indirect measure of disease severity. In addition, despite a small reduction in spleen weight (**Figure 2C**) and leukocyte number (**Figure 2D**) in B6.*Bach2*$^{\Delta T}$ mice, compared to control animals, this failed to reach a statistical difference at day 7 and 14 p.i.

We next investigated T cell responses to examine whether compensatory mechanisms develop in the absence of T cell BACH2 that may account for the lack of effect of BACH2-deificiency on disease outcome (**Figure 3A**). First, we found no statistically significant changes in the number of splenic CD4$^+$ or CD8$^+$ T cells in B6.*Bach2*$^{\Delta T}$ mice prior to infection (**Figure 3B**), although a small, consistent decrease in the frequency of these cell populations in the spleen was noted, compared to controls (**Figure 3C**). Following infection, splenic CD4$^+$ T cell numbers expanded in B6.*Bach2*$^{\Delta T}$ and control mice over the first 7 days of infection, but then declined over the following 7 days in B6.*Bach2*$^{\Delta T}$ mice, while continuing to increase in control animals (**Figure 3B**). Although no change in the number of B6.*Bach2*$^{\Delta T}$ Th1 and Tr1 cells was found, compared to controls, there was an increased frequency of both these cell populations over the course of infection, relative to B6.*Bach2*$^{fl/fl}$ cells. Given that CD8$^+$ T cells from B6.*Bach2*$^{\Delta T}$ also lack Bach2 expression, we measured these cells and found a similar pattern of expansion and contraction as seen with corresponding CD4$^+$ T cells (**Figures 3B,C**). Thus, despite significant changes in numbers and frequencies of B6.*Bach2*$^{\Delta T}$ T cell subsets following *P. chabaudi* infection, including increased frequencies of Th1 and Tr1 cells, there was little impact on disease outcome, suggesting that compensatory immune mechanisms were activated *in vivo*. Alternatively, it is possible that T cell-specific BACH2 is dispensable for control of parasite growth and disease outcome.

A Cell Intrinsic Role for BACH2 in Experimental Malaria

The above findings identify several important roles for BACH2 in T cell subset development and/or survival during experimental malaria. To test whether cell intrinsic roles for BACH2 in different T cell subsets were responsible, we generated B6.*Bach2*$^{\Delta T}$ (CD45.2):B6.*Bach2*$^{fl/fl}$ (CD45.1) (50:50) mixed bone marrow chimeric mice by injecting bone marrow from B6.*Bach2*$^{\Delta T}$ and B6.*Bach2*$^{fl/fl}$ mice into lethally irradiated B6.*Rag1*$^{-/-}$ mice (**Figure 4**). To generate animals with an approximate 50:50 mix of leukocytes, we had to increase the ratio of B6.*Bach2*$^{\Delta T}$:B6.*Bach2*$^{fl/fl}$ bone marrow to 70:30 because the former cells did not engraft as well as the control cells when injected in an equal ratio (**Figure 4A**). This result indicated a fundamental role for T cell BACH2 in the efficient reconstitution of immune systems following lethal irradiation. After 12 weeks of engraftment, similar B6.*Bach2*$^{\Delta T}$ and B6.*Bach2*$^{fl/fl}$ leukocyte reconstitution was measured (**Figure 4B**). However, despite the increased ratio of B6.*Bach2*$^{\Delta T}$ bone marrow in grafts resulting in relatively even leukocyte reconstitution from B6.*Bach2*$^{\Delta T}$ and B6.*Bach2*$^{fl/fl}$ bone marrow sources, we still found a reduction in B6.*Bach2*$^{\Delta T}$ CD4$^+$ T cells and CD8$^+$ T cell numbers in the spleens of uninfected mice, compared to controls (**Figure 4C**). This reduction was maintained following infection with *P. chabaudi* (**Figure 4C**). We next measured Th1 and Tr1 cell frequencies in mice over the course of *P. chabaudi* infection and found small reductions in *Bach2*-deficient, splenic Th1 and Tr1 cells at days 14 and 7 p.i., respectively (**Figure 4D**). Together, these results show that *Bach2* expression by T cells plays an important role in hematopoietic cell development, and in particular, the generation of CD4$^+$ and CD8$^+$ T cells. Furthermore, T cell intrinsic BACH2 is needed for efficient expansion of Th1 and Tr1 cells during experimental malaria.

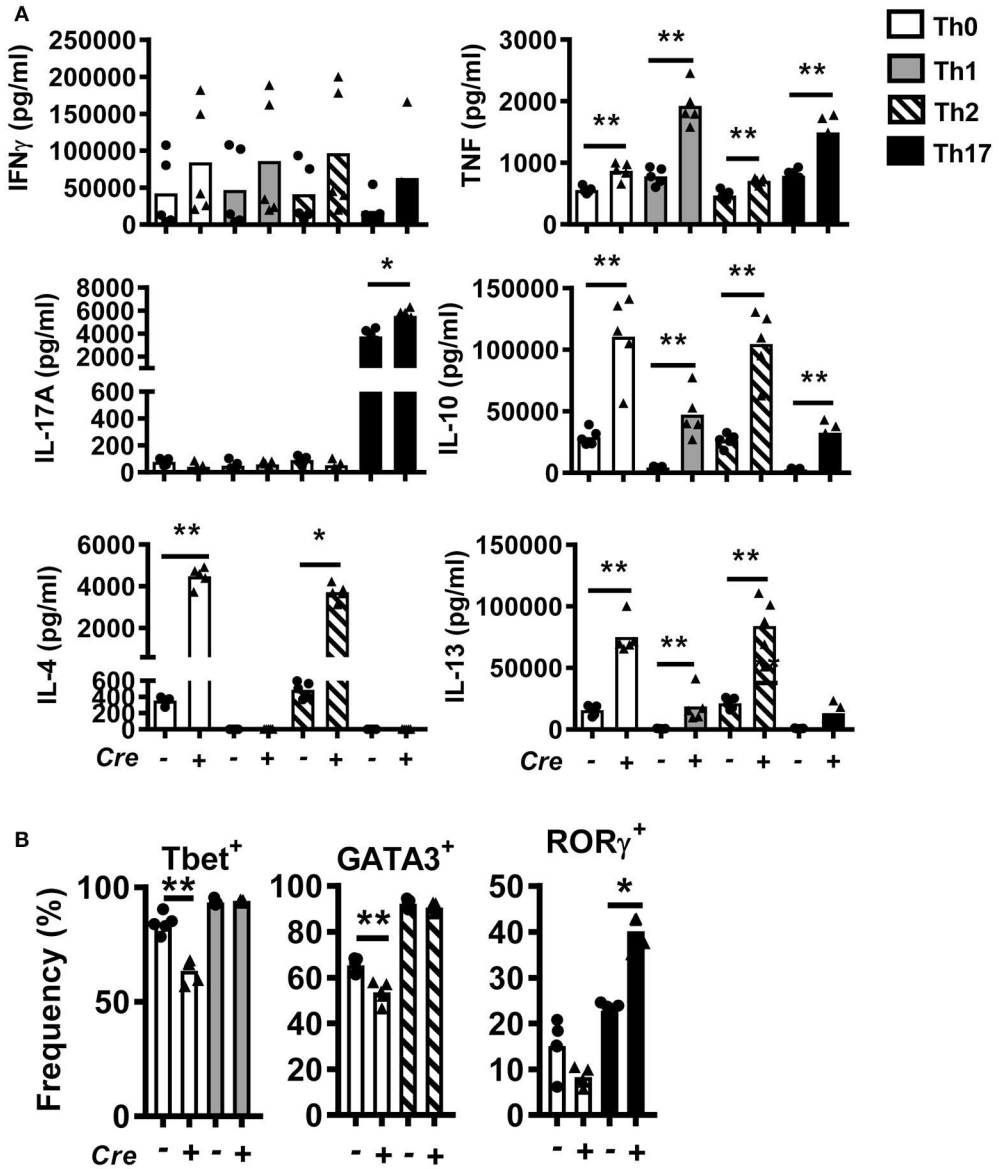

FIGURE 1 | Bach2 inhibits Th17 cell development *in vitro*. CD4$^+$ T cells were purified from B6.*Bach2*$^{\Delta T}$ (closed triangles) or B6.*Bach2*$^{fl/fl}$ (closed dots) spleens, as indicated, and cultured with αCD28 and αCD3 mAbs for 5 days under either Th0, Th1, Th2, or Th17 cell polarizing conditions. **(A)** Cytokine levels in cell culture supernatants were measured and **(B)** frequencies of CD4$^+$ T cells expressing lineage transcription factors under the various CD4$^+$ T cell polarizing conditions were also assessed. $n = 5$ mice per condition in duplicate, $^*P < 0.05$, $^{**}P < 0.01$, significance assessed by Mann-Whitney U-test.

This latter result was surprising given the increased expansion of Th1 and Tr1 cells in *P. chabaudi*-infected B6.*Bach2*$^{\Delta T}$ mice, compared to control mice, and indicates both cell extrinsic and intrinsic roles for BACH2 in Tr1 cell expansion during infection.

The Role of BACH2 in Experimental Visceral Leishmaniasis

The above results in experimental malaria were unexpected given the role of BACH2 in CD4$^+$ T cell subset development identified in *in vitro* experiments (**Figure 1**). Therefore, we also investigated the role of BACH2 in visceral leishmaniasis (VL) caused by infection with the human protozoan parasite *Leishmania donovani* to establish how broadly applicable our findings were. This C57BL/6J mouse model of VL is characterized by an acute, resolving infection in the liver, accompanied by the development of a chronic infection in the spleen (48, 49). Thus, in addition to examining immune responses in the spleen, we were also able to study these responses in the liver—a non-haematopoietic organ in adult mice. B6.*Bach2*$^{\Delta T}$ mice infected with *L. donovani* had little difference in spleen, liver or body weight, parasite burdens or leukocyte numbers over the first 28 days of infection,

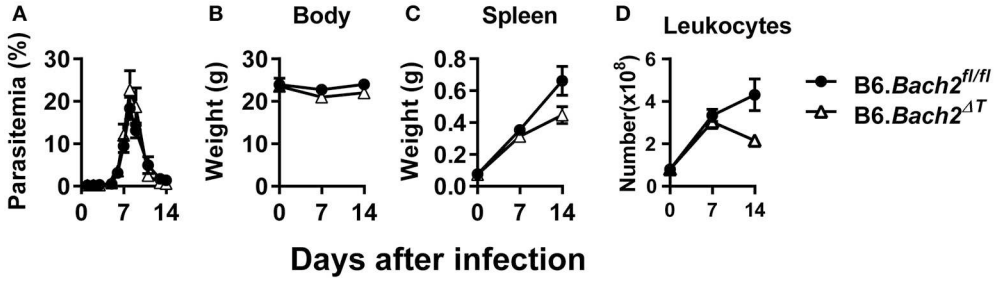

FIGURE 2 | T cell-specific BACH2 does not influence disease outcome in *P. chabaudi* infection. **(A)** Blood parasitemia was measured on days 1–8 p.i. ($n = 12$ mice per group), and days 9–14 p.i. ($n = 6$ mice per group). Whole body weights **(B)**, spleen weights **(C),** and numbers of splenic leukocytes **(D)** were measured in B6.*Bach2*$^{\Delta T}$ (open triangles) and B6.*Bach2*$^{fl/fl}$ (closed circles) mice infected with *P. chabaudi* at days 0, 7, and 14 p.i., $n = 5$–7 B6.*Bach2*$^{\Delta T}$ and $n = 5$ B6.*Bach2*$^{fl/fl}$ mice at each time point, mean ± SEM, significance assessed by Mann-Whitney U-test.

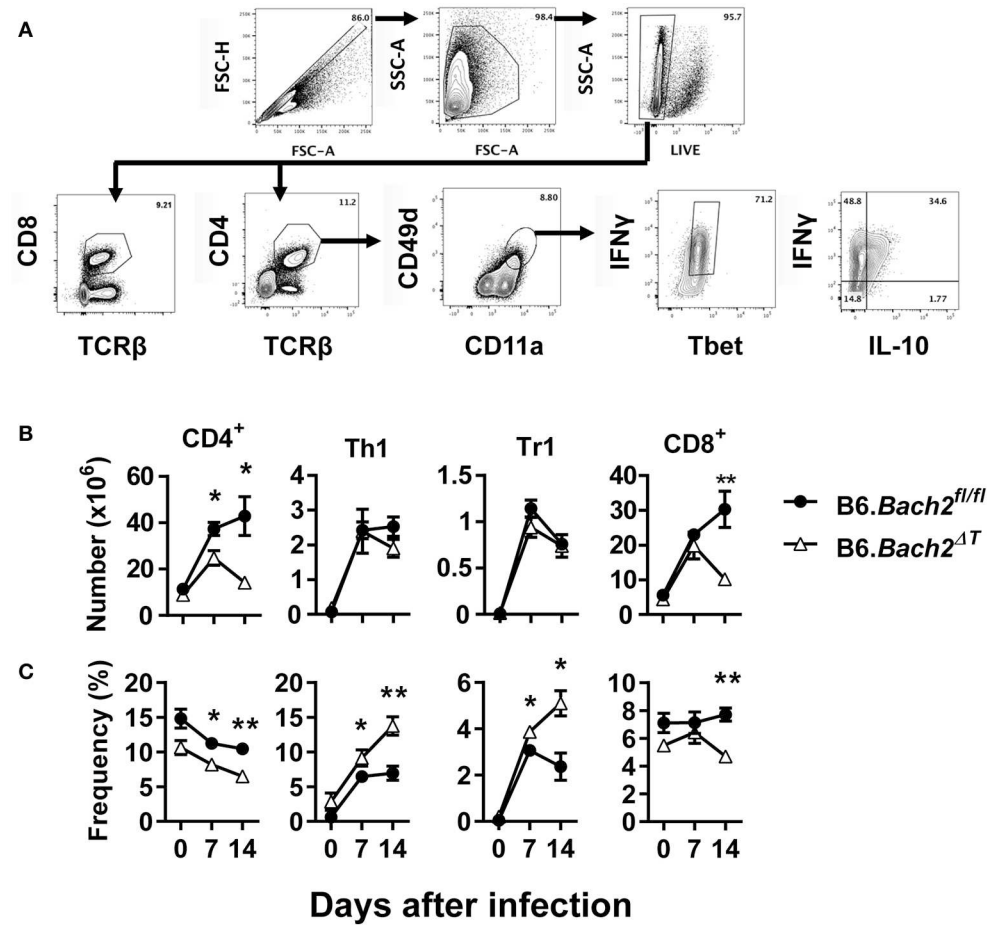

FIGURE 3 | T cell-specific BACH2 supports splenic CD4$^+$ T cell expansion. **(A)** Gating strategy for CD4$^+$ T, CD8$^+$ T, antigen experienced (CD49d$^+$ CD11a$^+$), Th1 (IFNγ$^+$, Tbet$^+$), and Tr1 (IFNγ$^+$ IL10$^+$) cells in the spleens of B6.*Bach2*$^{\Delta T}$ (open triangles) and B6.*Bach2*$^{fl/fl}$ (closed circles) mice infected with *P. chabaudi* at 0, 7, and 14 days p.i., Numbers **(B)** and frequency **(C)** of CD4$^+$ T, Th1, Tr1, and CD8$^+$ T cells, as indicated, were measured by flow cytometry. $n = 5$–7 mice per time point, mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$, significance assessed by Mann-Whitney U-test.

compared to control mice (**Figure 5**). Interestingly, although hepatic leukocyte numbers expanded in control B6.*Bach2*$^{fl/fl}$ mice over the first 14 days of infection, this was not observed in B6.*Bach2*$^{\Delta T}$ mice, which appeared to have higher leukocyte numbers in the liver prior to infection (**Figure 5C**).

Despite no significant changes in spleen and liver CD4$^+$ and CD8$^+$ T cell number or frequency (**Figures 6A–D**), we

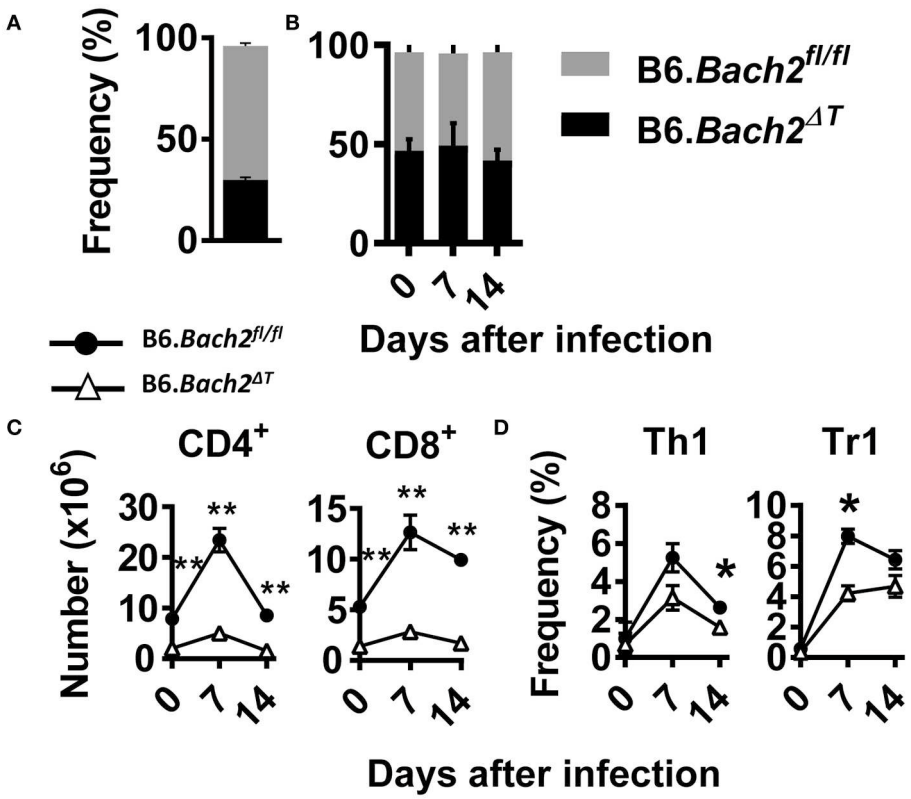

FIGURE 4 | Cell intrinsic BACH2 supports T cell development and expansion. B6.*Bach2*$^{\Delta T}$ (CD45.2):B6.*Bach2*$^{fl/fl}$ (CD45.1) (50:50) mixed bone marrow chimeric mice were generated in lethally irradiated B6.*Rag1*$^{-/-}$ mice and engraftment measured in splenic leukocytes 12 weeks later **(A)**. Subsequently, these mice were generated by injecting a 70:30 mix of bone marrow from *B6.Bach2*$^{\Delta T}$ and B6.*Bach2*$^{fl/fl}$ mice into irradiated B6.*Rag1*$^{-/-}$ mice, and the proportions of CD45.2 and CD45.1 leukocytes in the spleen were measured before *P. chabaudi* infection and 7 and 14 days p.i., as indicated **(B)**. Numbers of CD4$^+$ T and CD8$^+$ T cells **(C)**, as well as frequency of Th1 and Tr1 cells **(D)** in the spleen at days 0, 7, and 14 p.i., as indicated, were measured by flow cytometry. $n = 6$ mice per group per time point. Mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$, significance assessed by Mann-Whitney U-test.

did find tissue-specific changes in recently antigen-experienced (CD49d$^+$ CD11a$^+$) CD4$^+$ T cells in B6.*Bach2*$^{\Delta T}$ mice. There was a significant reduction in the frequency of recently antigen-experienced CD4$^+$ T cells at days 14 and 28 p.i., in the liver (**Figure 6E**), but not the spleen (**Figure 6C**), compared to B6.*Bach2*$^{fl/fl}$ mice. This finding suggests a role for BACH2 in the expansion and/or survival of activated CD4$^+$ T cells entering the liver during infection. However, despite these tissue-specific changes in recently antigen-experienced CD4$^+$ T cells, there was little impact of BACH2-deficiency on disease outcome in this second pre-clinical model of protozoan parasitic infection.

DISCUSSION

Our studies on BACH2 using T cell-specific knockout mice have demonstrated a T cell intrinsic role for BACH2 in T cell expansion and/or survival during *P. chabaudi* and *L. donovani* infection. These findings are consistent with those reported in other studies (2, 20, 50), and add to our understanding about the role of BACH2 in various CD4$^+$ T cell subsets. Our results indicate that T cell specific BACH2 deficiency most affects Th17 and Th2 cell development and maintenance. However, malaria and VL are not strongly influenced by these CD4$^+$ T cell subsets, which may explain why a relatively minor effect on disease outcome was found in pre-clinical models of these diseases. Thus, future studies on T cell BACH2 might best be directed toward disease where these CD4$^+$ T cell subsets are more important, such as multiple sclerosis or asthma, which are Th17 and Th2 cell-dependent, respectively. However, studies with mixed bone marrow chimeric mice also indicated both cell extrinsic and intrinsic roles for BACH2 in Th1 and Tr1 cell development, indicating that compensatory mechanisms may emerge in the absence of BACH2 during parasitic infection to initiate and maintain anti-parasitic immunity.

Our recent assessment of CD4$^+$ T cells from human peripheral blood showed that *BACH2* was down regulated during *P. falciparum* infection (51). This was consistent with results from other studies that indicate that *BACH2* needs to be down regulated to allow T cells to be activated and function effectively (2, 37, 50). Hence, another reason we may not have seen major changes in infected mice with *Bach2*-deficient T cells was because

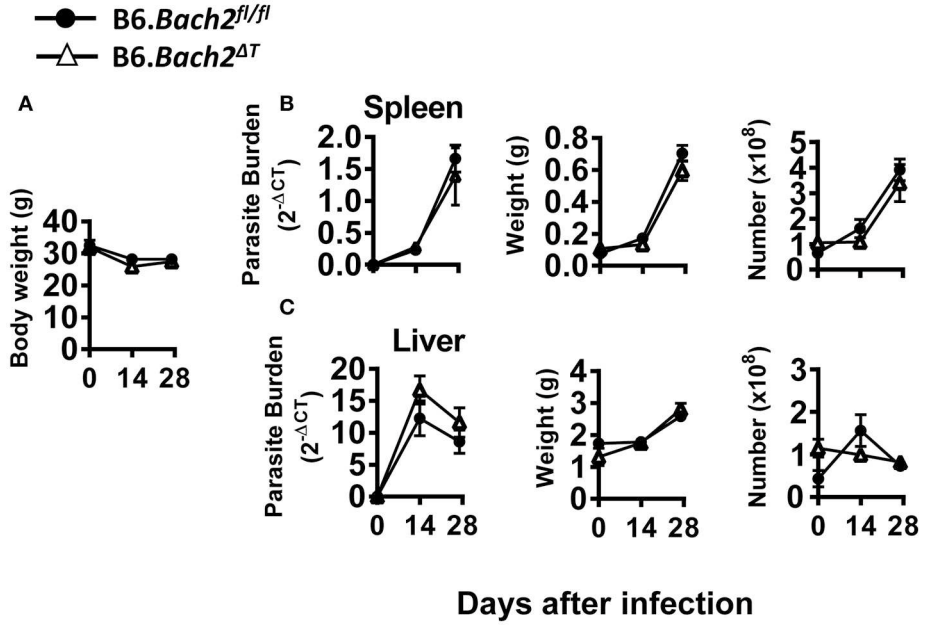

FIGURE 5 | T cell-specific BACH2 deficiency does not influence disease outcome in *L. donovani* infection. B6.*Bach2*$^{\Delta T}$ (open triangles) and B6.*Bach2*$^{fl/fl}$ mice (closed circles) mice were infected with *L. donovani* and assessed for body weight **(A)**, as well as spleen **(B)** and liver **(C)** parasite burden, organ weight and leukocyte number, as indicated, at days 0, 14, and 28 p.i., $n =$ 3–7 mice per group per time point, mean ± SEM.

gene expression was already down regulated. Any differences would likely occur during the initial response to infection, and therefore, studying earlier time points post-infection might reveal these effects. Also, given the down-regulation of *Bach2*, it may be informative to examine the impact of *Bach2* over-expression during infection in future studies.

We found that T cell-specific *Bach2* expression suppressed Th2 and Th17 cell development and/or activation. Similar results were observed in experiments involving cells from mice with ubiquitous *Bach2*-deficiency (2, 15). However, a problem with interpreting results from these experiments was the difficulty of excluding the possibility that *Bach2*-deficiency in a non-T cell population impacted T cell development, and was therefore responsible for changes observed. Our data indicate that T cell intrinsic BACH2 plays an important role in regulating Th2 cell cytokine production. However, other studies have shown that BACH2 also suppressed Th1 and Th17 cytokine production (2, 37). Our data supports this role in Th17 cells, but not in Th1 cell cytokine production.

Previous work has shown that BACH2 regulates BLIMP1 activity (52). Indeed, we recently showed that BLIMP1 was required for Tr1 cell development in experimental malaria and VL (28), and results from the current study showed increased frequencies of splenic Th1 and Tr1 cells in *P. chabaudi*-infected B6.*Bach2*$^{\Delta T}$ mice, despite reductions in overall CD4$^+$ T cells numbers, relative to littermate controls. However, as mentioned above, these increased Th1 and Tr1 B6.*Bach2*$^{\Delta T}$ cell frequencies were not observed in mixed bone marrow chimeric mice. Given that this latter setting is one where haematopoietic cells from B6.*Bach2*$^{\Delta T}$ and B6.*Bach2*$^{fl/fl}$ donors compete to fill various tissue niches and expand following infection, our results indicate an important role for BACH2 in T cell development, tissue recruitment and/or retention, independent of T cell activation, as well as a distinct role in CD4$^+$ T cell differentiation following infection. The balance between these different roles is likely to determine disease outcome in different settings, depending on the specific requirements of CD4$^+$ T cell subsets needed for protection. Therefore, although BACH2 may interact with BLIMP1 to influence CD4$^+$ T cell development and differentiation (52), our results indicate that this interaction plays a limited role in determining the outcome of infection with *P. chabaudi* or *L. donovani*.

As mentioned earlier, BACH2 impacts the terminal differentiation of CD8$^+$ T cells by controlling availability of transcription factor binding sites, and in particular, by controlling AP-1 availability (50). It is possible that BACH2 acts in a similar manner to influence development of different CD4$^+$ T cell populations. NF-kB and AP-1 family members are required for full Th2 cell differentiation, and the Th2 cell cytokines IL-4 and IL-13 have AP-1 binding sites in their promoters (53, 54). Thus, BACH2 may inhibit the expression of these genes by preventing AP-1 binding, and therefore prevent their transcription and subsequent expression. Support for this mechanism came from studies using an AP-1 decoy molecule, which was used to block IL-4 and IL-13 production, and ameliorate disease symptoms in a model of asthma (55). NF-kB/AP-1 binding was also increased in rheumatoid arthritis patients (56), and similarly, *NF-kB/AP-1* expression

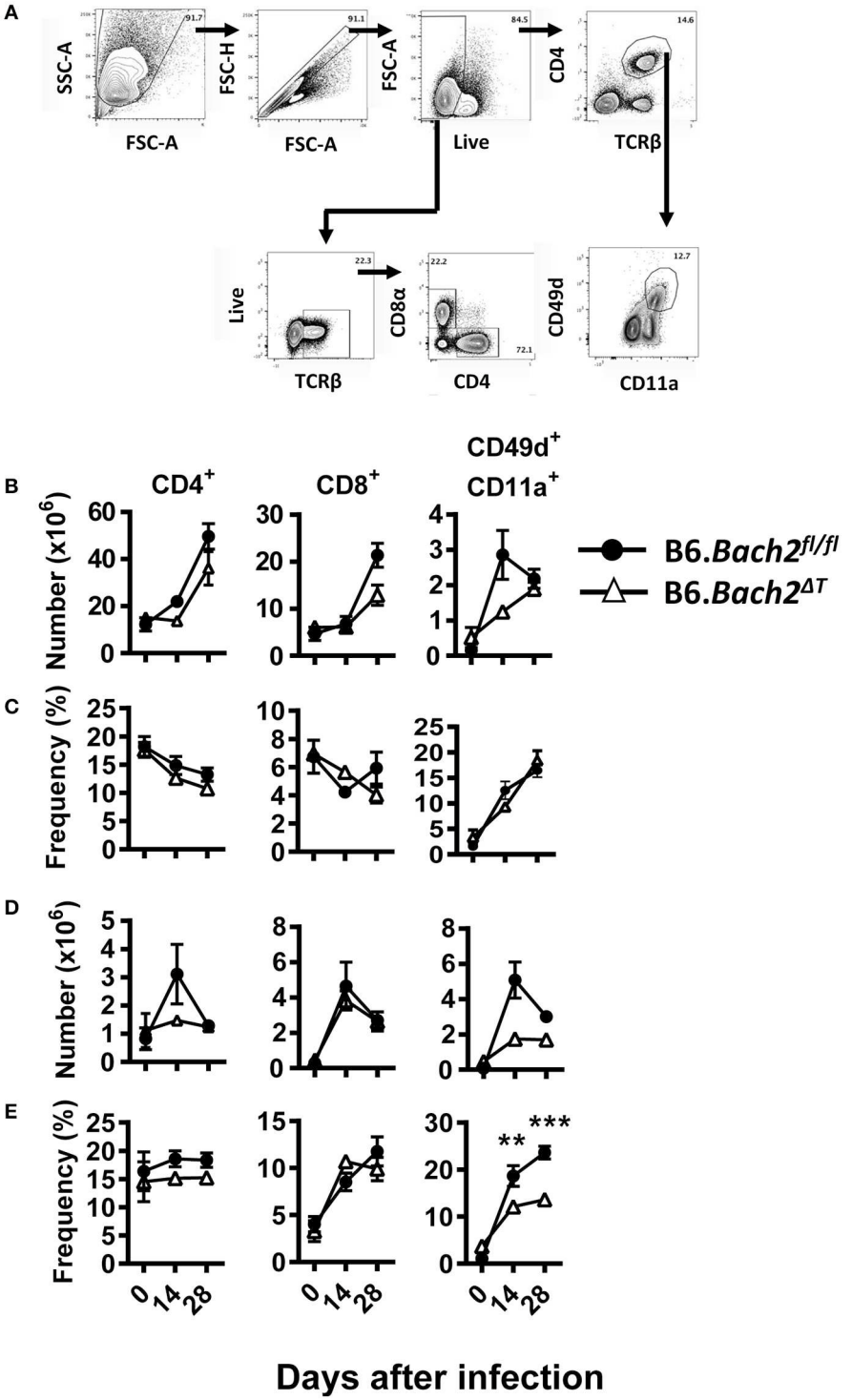

FIGURE 6 | T cell-specific BACH2 influences tissue-specific expansion of antigen experienced $CD4^+$ T cells following *L. donovani* infection. **(A)** Gating strategy for $CD4^+$ T, $CD8^+$ T, and antigen-experienced ($CD49d^+$ $CD11a^+$) $CD4^+$ T cells in the spleen **(B,C)** and liver **(D,E)** of B6.*Bach2*$^{\Delta T}$ (open triangles) and B6.*Bach2*$^{fl/fl}$ (closed circles) mice infected with *L. donovan* at 0, 14, and 28 days p.i., The numbers **(B,D)** and frequencies **(C,E)** of $CD4^+$ T, $CD8^+$ T, and antigen-experienced $CD4^+$ T cells, as indicated, were measured by flow cytometry. $n = 4$–7 mice per time point, mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$ significance assessed by Mann-Whitney U-test.

was correlated with type-1 diabetes pathogenesis (57). Given that both diseases are associated with *BACH2* dysregulation (8, 58), this may contribute to aberrant *NF-kB/AP-1* expression. Others also hypothesize that the homology between BACH2 and AP-1 sequences may allow BACH2 to bind in place of AP-1 in umbilical cord blood, where *BACH2* was shown to regulate IL-2 expression (59). Therefore, one mechanism by which BACH2 may influence T cell differentiation and disease outcome is by competing with AP-1 for DNA binding.

The absence of T cell BACH2 may also promote changes in the regulation of apoptosis, as the JNK/AP-1 pathway has been associated with apoptosis in synovial cells in rheumatoid arthritis (60), and several studies have implicated BACH2 in promoting apoptosis. For example, BACH2 facilitated apoptosis in B cells by suppressing anti-oxidative and anti-apoptotic genes (61, 62). However, the loss of BACH2 caused enhanced $CD8^+$ T cell apoptosis 5–10 days after viral infection (50), indicating a different role in T cells. This may help explain the loss of *Bach2*-deficient $CD4^+$ T cells we observed in the spleen during *P. chabaudi* infection, although this remains to be tested. Furthermore, apoptosis was found to be associated with a reduction in anti-apoptotic Bcl-2 family proteins Bcl-xL and Mcl-1 (50). Interestingly, BACH2 has been associated with Bcl-2 and Mcl-1 in other disease settings (58). Therefore, although BACH2 is pro-apoptotic in B cells, it appears to act as an anti-apoptotic molecule in $CD8^+$ T cells, and if it has a similar role in $CD4^+$ T cells, this may help to explain some of our findings.

In summary, we showed BACH2 is an important intrinsic factor in $CD4^+$ T cells during differentiation *in vitro* and during parasitic infection. We showed that T cell-specific BACH2 modulates Th2 and Th17 cell differentiation, and suppressed effector $CD4^+$ T cell responses during infection. However, BACH2 was especially important for the expansion and/or maintenance of splenic $CD4^+$ T cells during *P. chabaudi* infection, and a possible mechanism for this role may be via regulation of AP-1 binding to cell lineage-specific genes and anti-apoptotic genes. Our results indicate that BACH2 either has a minor role in disease outcome during malaria and VL or compensatory mechanisms for BACH2 function are effectively activated following infection with the protozoan parasites that cause these diseases.

ETHICS STATEMENT

All animal procedures were conducted with the approval of the QIMR Animal Ethics Committee under the animal ethics number A02-634M and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian NHMRC, Canberra).

AUTHOR CONTRIBUTIONS

CLE and CRE designed, performed and analyzed the work, and wrote the paper. MdO, FdLR, RK, SN, YW, and FA performed the work and analyzed the data. KK, TK, TS, and AK provided reagents and expert advice on experimental design and interpretation of data.

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TACI Contributes to *Plasmodium yoelii* Host Resistance by Controlling T Follicular Helper Cell Response and Germinal Center Formation

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The delay in parasite-specific B cell development leaves people in malaria endemic areas vulnerable to repeated *Plasmodium* infections. Here, we investigated the role of transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), a molecule involved in the generation of antigen-specific antibody secreting cells, in host response to non-lethal *Plasmodium yoelii* infection. We found that TACI deficiency not only resulted in higher peak parasitemia levels in *P. yoelii* challenged mice, but also led to a delay in parasite clearance and anti-*P. yoelii* Merozoite Surface Protein 1(C-terminal 19-kDa fragment [rMSP-1_{19}]) protein and anti-rMSP-1_{19} and anti-*P. yoelii* IgG antibody development. There was also a delay in the generation of splenic high affinity antibody secreting cells that recognize rMSP-1_{19} protein as compared to wild-type mice. Interestingly, coinciding with the delay in parasite clearance there was a delay in the resolution of T follicular helper (T_{FH}) cell and germinal center (GC) B cell responses in TACI -/- mice. The persistence of T_{FH} and GC B cells is likely a result of enhanced interaction between T_{FH} and GC B cells because inducible costimulator ligand (ICOSL) expression was significantly higher on TACI -/- GC B cells than wild-type cells. The difference in the kinetics of GC reaction appeared to also impact the emergence of plasma cells (PC) because there was a delay in the generation of TACI -/- mice PC. Nevertheless, following the recovery from *P. yoelii* infection, TACI -/- and wild-type mice were both protected from a rechallenge infection. Establishment of protective B cell response was responsible for the resolution of parasitemia because B cells purified from recovered TACI -/- or wild-type mice were equally protective when introduced to naïve wild-type mice prior to *P. yoelii* challenge. Thus, despite the increased susceptibility of TACI -/- mice to *P. yoelii* infection and a delay in the development of protective antibody levels, TACI -/- mice are able to clear the infection and resist rechallenge infection.

Keywords: *Plasmodium yoelii*, TACI, T follicular helper cell, Germinal center, B cell, antibody

INTRODUCTION

Children under the age of 5 years (1), especially those who are less than 1 year of age, are highly vulnerable to *Plasmodium* infections (2). While antibodies play a critical role in controlling parasitemia burden and illness (3), protective humoral immunity to malaria occurs only after repeated exposure to parasites (4). Shortcomings of immunological response that can control *Plasmodium* parasites have been attributed to the diversity of the malarial antigens, the rapid disappearance of anti-malarial antibodies and an insufficient long-lived plasma cell (PC) pool (4). Despite the recognition of these B cell insufficiencies, molecular and cellular events that prevent the host's ability to mount optimal B cell responses are poorly understood.

In this study, we examined the role of transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) in host resistance to malaria infection. TACI is a receptor for B cell activating factor belonging to TNF family (BAFF) and a proliferation-inducing ligand (APRIL) (5). Together with two other receptors, BAFF receptor (BAFF-R) and B cell maturation antigen (BCMA), these molecules are crucial in maintaining B cell homeostasis, and TACI is involved in immunoglobulin isotype switching and antibody secretion, PC maintenance and macrophage polarization (6–10). TACI is also important in controlling T follicular helper (T_{FH}) cell responses as immunization or infection of TACI deficient mouse results with augmented T_{FH} development (11, 12). However, while immunization of TACI -/- mice with a T cell dependent antigen elicited reduced antibody responses and short lived PC as compared to wild-type mice (11), TACI -/- mice controlled *Citrobacter rodentium* infection better than the wild-type mice most likely because of an increase in antibody secreting cells and development of high affinity antibodies directed against *C. rodentium* (12). Measurement of elevated circulating BAFF and increased BAFF-R on B cells in humans experimentally challenged with *Plasmodium falciparum* suggest an involvement of these molecules in host response to malaria (13, 14). Whether TACI participates in BAFF-induced host responses during malaria infection has not been explored.

We found that *Plasmodium yoelii* challenged TACI -/- mice manifested significantly higher levels of parasitemia than wild-type mice, which persisted longer. The increased susceptibility of TACI -/- mice appeared to be the result of a delay in anti-parasite antibody development. Analysis of T_{FH} cell development and germinal center (GC) formation suggested that altered kinetics of GC reaction may be responsible for the delay in the PC development and antibody production in infected TACI -/- mice. Nevertheless, despite late parasite clearance, not only were the TACI -/- mice protected from a second *P. yoelii* challenge, but also, B cells from TACI -/- mice were sufficient to prevent *P. yoelii* infection when transferred to naïve wild-type mice. In the absence of TACI, host control of parasitemia is delayed compared to wild-type mice. However, once the parasitemia is cleared, B cell mediated immunity renders TACI -/- mice resistant to a second infection.

MATERIALS AND METHODS

Mice

C57BL/6 mice (6–8 weeks old) were purchased from the Jackson Laboratories (Bar Harbor, ME). TACI -/- mice on a C57BL/6 background were described previously (15, 16). The experimental procedures were approved by the Institutional Animal Care and Use Committee of the Center for Biologics Evaluation and Research (Protocol-2008-08).

Parasites

Nonlethal *P. yoelii* strain 17XNL was used in mouse challenge experiments (17). Frozen stocks of *P. yoelii* 17XNL-infected erythrocytes were intraperitoneally (i.p.) injected to C57BL/6 mice to generate donor mice. When 8 to 10% parasitemia was detected, blood was collected by cardiac puncture, diluted in PBS and used to i.p. infect experimental animals with 1×10^6 *P. yoelii* parasites in 200 μl of PBS. The percent parasitemia [parasitized red blood cells (RBCs)/total RBCsx100] after infection was determined by Giemsa-stained thin blood smears.

Anti-Plasmodium Antibody Detection

Serum samples were pooled or not from 3 C57BL/6 and 3 TACI -/- mice per time point at 8, 16, 22, 28, and 71 days post *P. yoelii* infection. Serum antibody levels against an extended version of the *P. yoelii* Merozoite Surface Protein 1(C-terminal 19-kDa fragment [rMSP-1_{19}]) (18) and whole *P. yoelii* 17XNL extract were measured by ELISA. ELISA plates were coated with 70 ng/well of rMSP-1_{19} or sonicated *P. yoelii* infected RBCs in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$). After washing with PBS/0.05%Tween-20, plates were blocked with 5% milk/PBS. Next, 100 μl of 1:50 to 1:51200 titrated sera were added. After 2 h, plates were incubated with 1:3500 diluted goat anti-mouse IgG-HRP antibody (Southern Biotechnology Associates, Birmingham, AL). Plates were read on a VERSA max microplate reader after adding ABTS substrate (Molecular Devices, Sunnydale, CA).

Adoptive Transfer of B Cells

Splenic B cells of mice that had cleared *P. yoelii* infection 4 months earlier were isolated by using B220 magnetic beads (Miltenyi Biotec, San Diego, CA). The purity of $B220^+CD19^+$ B cells was > 95%. *Plasmodium yoelii* immune B-cells were then adoptively transferred by intravenous (i.v.) injection of 3×10^7 immune cells per mouse. Two hours after the B cell transfer, mice were injected with *P. yoelii* infected RBCs. The percent parasitemia (parasitized RBCs/total RBCs × 100) after infection was determined by examining Giemsa-stained thin blood smears.

Flow Cytometry

Single cell suspensions prepared from spleen and dead cells were excluded after staining with fixable efluor 780 (Affymatrix, Santa Clare, CA). For T_{FH} analysis, splenocytes were stained in 2%FBS/0.5 EDTA/PBS buffer with anti-CD4-PercpCy5.5 (clone GK1.55, Affymatrix), PD-1-PE (clone 29F.1A12, BioLegend, San Diego, CA), CD44-Alexa Fluor 700 (clone IM7, BD Biosciences, San Jose, CA), CXCR5-biotin (clone 2G8, BD Biosciences), and ICOSL-biotin (clone HK5.3, Biolegend) antibodies. Biotin

was detected with streptavidin-BV421 (BioLegend). For GC B cell and PC analysis, B220-BV605 (clone RA3-6B2, BioLegend), FAS-APC (clone J02, BioLegend), T/B cell activating antigen-FITC (clone GL-7, BioLegend), B220-APC (clone RA3-6B2, BioLegend), CD138-PE (clone 281-2 from BioLegend), and IgD-BV605 (clone 11-26c.2a, BD Biosciences) antibodies were used. For intracellular staining, samples were fixed with the Foxp3 Fix/Perm buffer set, following the manufacturer's instructions (eBioscience). Samples were then intracellularly stained with α-Foxp3 (BioLegend, 150D, 1:100) antibody. 'Fluorescence minus one' (FMO) controls were used to gate the cells for each antibody. Data were acquired on LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software v10 (FlowJo, Ashland, OR).

Immunofluorescence Microscopy

OCT-embedded spleens were snap-frozen by floating on liquid nitrogen-cooled isopentane. Frozen tissues were cut into 10 μm sections using a cryostat and mounted on poly-lysine-coated slides. Sections were allowed to air dry at room temperature for 10 min and fixed with pre-cooled Acetone and Methanol (1:1 vol) for 10 min, followed by washing three times with PBS containing 0.5% Tween-20. Sections were first blocked with 5% goat serum for 1 h, and then stained overnight at 4°C with anti-B220 (Rat IgG, 1:50, eBioscience), anti-GL-7 (Rat IgM, 1:50, BioLegend) antibodies. Following three washing steps with PBS containing 0.5% Tween-20, sections were stained for 1 h at room temperature with the following secondary antibodies: goat anti-rat IgG-AF488, goat anti-rat IgM-AF647 (1:200, Invitrogen, Carlsbad, CA). Samples were imaged on Leica DMI6000 Inverted Light Microscope. The tile scan function was used to stitch individual 10x images together.

Elispot Assay

rMSP-1_{19}-specific antibody secreting cells (ASC) were quantified by direct *ex vivo* ELISPOT assay. Ninety-six-well nitrocellulose plates (Multiscreen-HA; Millipore, Bedford, MA) were coated overnight at 4°C with rMSP-1_{19} at 10 μg/ml concentration and incubated in 15 μM 2-mercaptoethanol/10%FBS/RPMI for 1 h. Splenocytes or bone marrow cells (10^6 cells/well/100 μl) were incubated at 37°C, 5% CO_2 for 5 h in RPMI medium. Plates were washed, and bound rMSP-1_{19}-specific IgG-producing cells were stained with goat anti-mouse IgG-HRP (Bethyl Laboratories, Montgomery, TX) antibody. HRP was developed with 3-aminoethyl carbazole, AEC peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Spots were counted using an AID ELISPOT analyzer (Autoimmun Diagnostika, Germany).

Antibody Avidity Measurement

Serum samples collected from 5 C57BL/6 mice and 5 TACI -/- mice 71 days after *P. yoelii* infection were pooled. The avidity of the antibodies was evaluated using guanidine hydrochloride (GuHCl) as a dissociative agent (19). The ELISA plates were coated, blocked and serum titrations were prepared as described in ELISA method. Triplicate serum samples were added to each well. After 2 h of incubation and washing steps, "avidity samples" were incubated with 100 μl of 0.1 M GuHCl (Sigma, Darmstadt, Germany) while triplicate "control samples" were incubated in washing buffer. After incubation for 10 min and 5 washes, wells were exposed to goat anti-mouse IgG-HRP antibody (Southern Biotechnology Associates) for 1 h. Following washing steps, ABTS substrate was added and the plates were read on a VERSA max microplate reader (Molecular Devices). Antibody avidity was calculated using the method described by Perciani et al. (20). Optical densities from each titration were graphed using GraphPad Prism software (La Jolla, CA) and the area under the curve (AUC) was measured for both the GuHCl and control-treated samples for each serum pool. The formula (AUC of guanidine treated samples)/(AUC of control-treated samples) was used to calculate the avidity index ratio.

Statistical Analyses

The parasitemia data were evaluated using unpaired Student's *t*-test. Unpaired Student's *t*-test was also used for the comparison of cell numbers measured in flow cytometry and ELISPOT analysis. Values of $p < 0.05$ were considered statistically significant. The avidity ELISA data was evaluated using AUC with a baseline value of 0, and the means compared using a Mann-Whitney non-parametric test.

RESULTS

Elevated Parasitemia and Delayed Clearance of *P. yoelii* in TACI -/- Mice

To assess whether TACI participates in resistance to *Plasmodium* infection, we challenged C57BL/6 and TACI -/- mice with *P. yoelii*. Parasitemia levels were monitored by counting the percentage of *P. yoelii* infected RBCs until the resolution of parasitemia. The wild-type mice developed a typical self-limiting course of parasitemias that characterize infections with the non-lethal *P. yoelii* strain 17XNL (21), with peak parasitemia on day 11 post-infection, and parasitemia resolution by day 21 post-infection (**Figure 1A**). In contrast, parasitemia levels were significantly higher in TACI -/- mice starting at day 11 post-*P. yoelii* infection when compared to the wild-type mice. Parasitemias continued to increase in TACI -/- mice after day 11 post-infection, until reaching a peak at day 21 post-infection. In contrast, C57BL/6 mice had 0% parasitemia at day 21 post-infection. Thus, in the absence of TACI, malaria parasite load was increased, and parasitemia resolution was markedly delayed.

Anti-*Plasmodium* Antibody Responses Are Delayed in TACI -/- Mice

Antibodies to *Plasmodium* are involved in reducing parasite load and clearance in both mice and humans (3, 22). To assess whether antibody development was altered in parallel to the delay of parasitemia resolution in TACI -/- mice, serum antibodies against rMSP-1_{19}, a surface exposed fraction of *P. yoelii* MSP-1 protein that is a target of protective antibodies (18), were measured over the course of the infection. Both strains had low levels of anti-rMSP-1_{19} IgG antibodies on day 8 post-infection (**Supplementary Figure 1A**). Although anti-rMSP-1_{19} IgG levels increased thereafter in both strains, wild-type mice levels were markedly higher on days 16 and 22 as compared to those of TACI -/- mice (**Figure 1B**). TACI -/- mice antibody levels

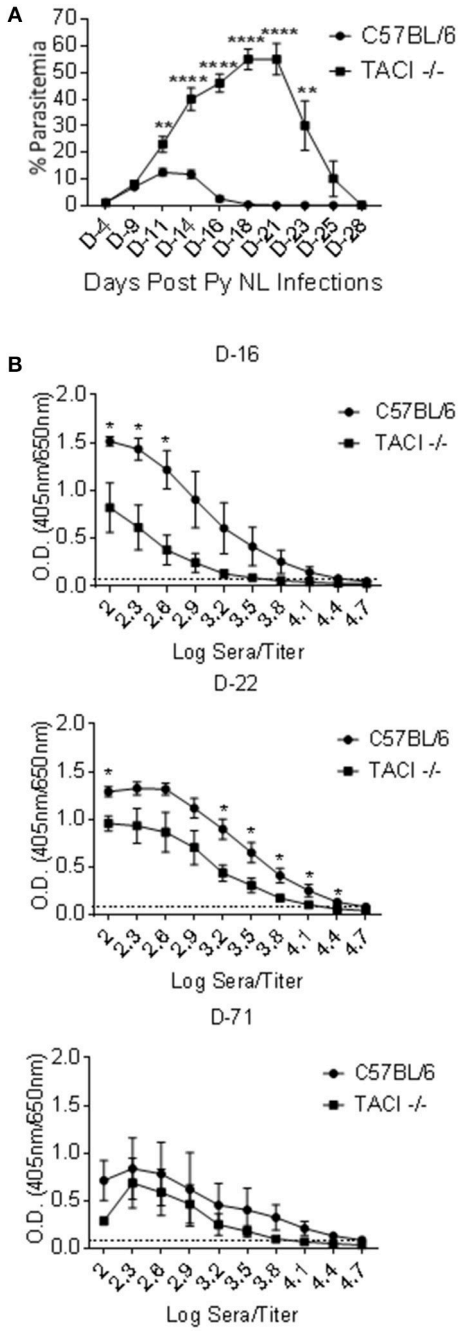

FIGURE 1 | *P. yoelii* infection clearance and antibody development are delayed in TACI -/- mice. **(A)** TACI -/- and C57BL/6 mice were infected (i.p.) with 1×10^6 *P. yoelii* (Py 17XNL) parasites. Parasitemia levels were evaluated by blood smears at indicated days post challenge until parasitemia clearance. Results are expressed as mean % parasitemia ± SEM for 10 mice per group. One representative experiment out of three with similar infection outcome is shown. **(B)** TACI -/- and C57BL/6 mice (9 per group) were infected (i.p.) with 1×10^6 *P. yoelii* parasites. Individual serum was collected from three mice of each group (per time point) on days 16, 22, and 71 post parasite challenge and were tested in ELISA for anti-rMSP-1_{19} IgG antibody measurement (Please see **Supplementary Figure 1A** for days 8 and 28 ELISA results). Average OD values ± SEM from three samples on each time point were plotted. Dotted line indicates ELISA negative control. Unpaired student's *t*-test was used for statistical evaluation. *indicates $p < 0.05$, ** < 0.01; ****$p <$ 0.0001. Experiment was performed once.

reached comparable levels to those elicited in the wild-type mice only on day 28 (**Supplementary Figure 1A**) and remained at levels comparable to those of wild-type mice at 71 days post infection (**Figure 1B**). Similar trends in antibody responses were observed when sera were tested for IgG antibodies against *P yoelii* parasites (**Supplementary Figure 1B**). Since the peak anti-*P. yoelii* antibody development coincided with the parasitemia resolution time-point in TACI -/- mice, this delayed antibody response is likely responsible for the magnitude and the delay in the resolution of parasitemia.

TACI Deficiency Extends Formation and Resolution Kinetics of the GC Response

T_{FH} cells provide soluble and contact-dependent signals to B cells for somatic hypermutation and affinity maturation of antibodies in the GC (23). Following T_{FH} help, activated B cells leave the GC as plasmablasts and memory B cells (24). Moreover, the formation of T_{FH} cells and their communication with GC B cells is required for the development of parasite-specific B cells during *Plasmodium* infection (11, 25). The discovery of delayed kinetics of anti-malarial antibody development in *P. yoelii* infected TACI -/- mice led us to investigate the T_{FH} development and GC formation in infected mice spleens. In wild-type mice, the number of $CD4^+CD44^+CXCR5^{high}PD\text{-}1^{high}$ T_{FH} cells (**Supplementary Figure 2A**) were highest at day 10 post-infection, which steadily declined on days 15 and 23 (**Figures 2A,B**). The percentage of T_{FH} cells in wild-type mice followed kinetics similar to the number of T_{FH} cells (**Supplementary Figure 2B**). Compared to T_{FH} cells, wild-type mice $B220^+GL7^+FAS^+$ (**Supplementary Figure 2A**) GC B cell-percentages and numbers peaked slightly later (day 15) (**Figures 2C,D**). In TACI -/- mice, day 10 T_{FH} cell-numbers were comparable to those of wild-type mice on the same day (**Figures 3A,B**). However, instead of declining thereafter as in wild-type mice, TACI -/- T_{FH} cell-numbers persisted until day 15. The kinetics of TACI -/- mice T_{FH} cell-percentage was similar to the changes in T_{FH} cell numbers (**Supplementary Figure 2B**). This delayed resolution of T_{FH} cells coincided with a significantly more GC B cell numbers in TACI -/- mice than wild-type mice on day 15 (**Figures 2C,D**). Both mouse strains had lower numbers of GC B cells on day 23 than on day 15, but TACI -/- mice cell numbers were still significantly more than those of wild-type mice. Similar to the number of GC measured on day 15, the percentage of GC B cells on the same day were significantly higher in TACI -/- mice than wild-type mice, but the difference between the two mouse strains vanished on day 23 (**Supplementary Figure 2C**).

Although the $CD4^+CD44^+CXCR5^{high}PD\text{-}1^{high}$ cells were initially designated as T_{FH} cells that promote B cell responses in the GC (23), more recent studies showed that a Foxp3-expressing regulatory subset of T_{FH} cells (T_{FR}) is inhibitory to the GC reaction and B cell responses (26, 27). The function of these cells can be assessed by analyzing the ratio of T_{FR} to T_{FH} (T_{FR}:T_{FH}) cells, which predicts the influence of T_{FR} cells on antibody responses (28). Since we measured lower anti-parasite antibody levels despite the persistent of T_{FH} and GC in TACI -/- mice, higher T_{FR}:T_{FH} ratio could be responsible for the ablated antibody responses. However, this

possibility was ruled out because we measured comparable T_{FR}:T_{FH} ratios between the mouse strains at 15 day time point (**Supplementary Figures 3A–C**).

Interaction of ICOSL on B cells with ICOS on pre-T_{FH} cells is essential for early stage T_{FH} cell formation (29). In nitrophenyl-chicken gamma globulin immunized TACI -/- mice, the elevated ICOSL expression has been implicated in expanded T_{FH} and GC formation (11, 12). Reminiscent of immunized and *C. rodentium* infected TACI -/- mice, we also detected significantly higher ICOSL expression on TACI -/- B cells as compared to wild-type mice at 10 days post-infection (**Figure 2E**). Thus, the elevated ICOSL on GC B cells likely contributes to the persistent T_{FH} and GC B cell interaction in *P. yoelii* infected TACI -/- mice.

The Emergence of PC Is Delayed in TACI -/- Mice

Although an increase in ICOSL expression on B cells has been detected in both immunized and *C. rodentium* infected TACI -/- mice, the consequence of elevated ICOSL on PC development and antibody response was different in the two studies (11, 12). In immunized TACI -/- mice elevated ICOSL resulted in impaired PC development and reduced antibody responses (11). In contrast, *C. rodentium* infection of TACI -/- mice resulted in higher avidity antibodies that cleared the infection faster than wild-type mice did (12). To assess whether the persistence of GC for an extended duration together with increased ICOSL expression on TACI -/- GC B cells impacted the development of PC exiting the GC, we analyzed the kinetics of PC formation

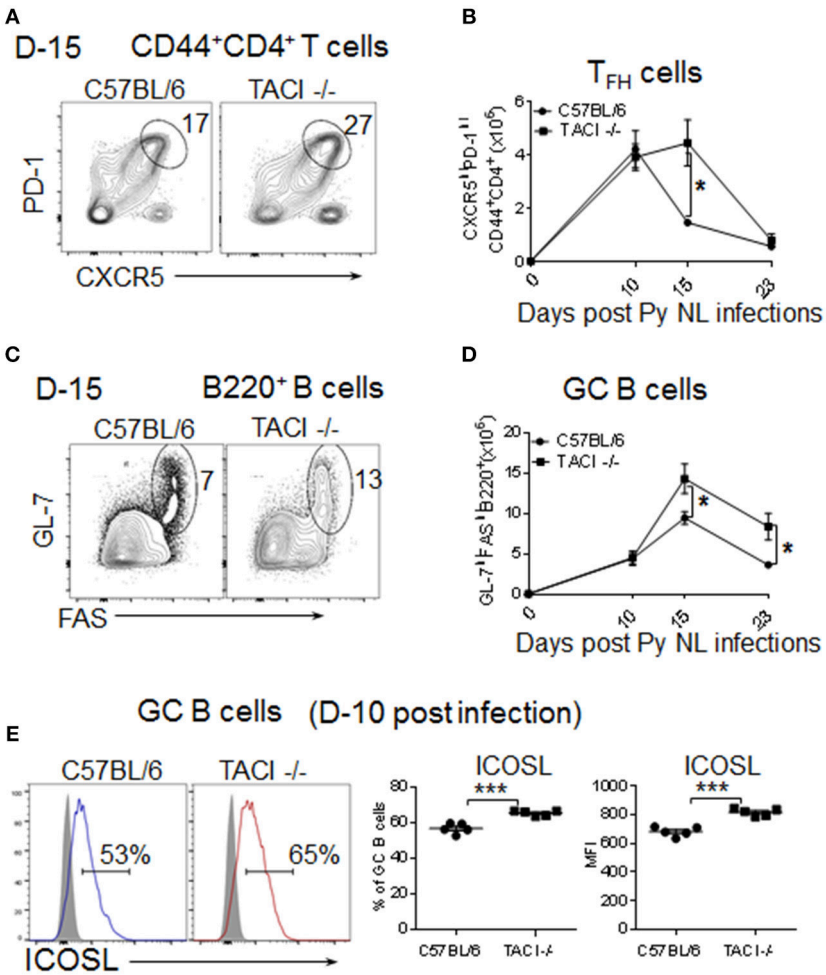

FIGURE 2 | TACI deficiency extends formation and resolution kinetics of T_{FH} and GC response. TACI -/- and C57BL/6 mice were infected (i.p.) with 1×10^6 *P. yoelii* (Py 17XNL strain) parasites. **(A)** Representative dot plots depict the percentage of splenic PD-1^{high}CXCR5^{high} (T_{FH}) cell on CD44+CD4+ pre-gated T cells at day 15 post-infection. **(B)** Formation and resolution kinetics of T_{FH} cells presented as number of T_{FH} cells per spleen. **(C)** Representative dot plots depict the percentage of and GL-7^{hig}hFAS^{high} (GC B cells) on B220$^+$ pre-gated B cells at day 15 post-infection. **(D)** Formation and resolution ki"netics of GC B cells presented as number of GC B cells per spleen. Total splenic B cell **(E)** Day 10 post-infection ICOSL expression levels were measured on B220$^+$GL-7^{high}FAS^{high} gated splenic GC B cells. Representative histograms as well as frequencies of ICOSL expressing cells and ICOSL MFI for each mouse strain are shown. Unpaired Student's *t*-test was used for statistical evaluation. Results are expressed as mean ± SEM ($n = 5$) from one representative experiment out of three with similar results. $^*p < 0.05$ and $^{***}p < 0.001$ for TACI -/- vs. C57BL/6 comparison. GC, germinal center.

and GC development in the spleens of TACI -/- and wild-type mice following *P. yoelii* infection. As expected, both mice were free from GC occupying B cell follicles on day 8 post-infection (**Figure 3A**). We observed more PC ($B220^{low}GL\text{-}7^{+}$) located in the marginal zones of wild-type mice spleens than the marginal zones of TACI -/- mice, which suggested the initiation of T-cell independent response (30) as early as day 8 in wild-type mice because, together with B1 cells, marginal zone B cells are responsible for T-cell independent responses (31). The absence of PC ($B220^{low}GL\text{-}7^{+}$) in TACI -/- mice marginal zones is consistent with the well-established role for TACI in mediating T-cell independent responses (16, 32). At the same time, TACI -/- mice T cell zone was already populated with activated GL-7^{+} cells (33), suggesting that T cells were activated faster in TACI -/- than in wild-type mice (**Figure 3A**). Both strains had well-formed GC ($B220^{+}GL\text{-}7^{+}$) (34, 35) in B cell follicles as

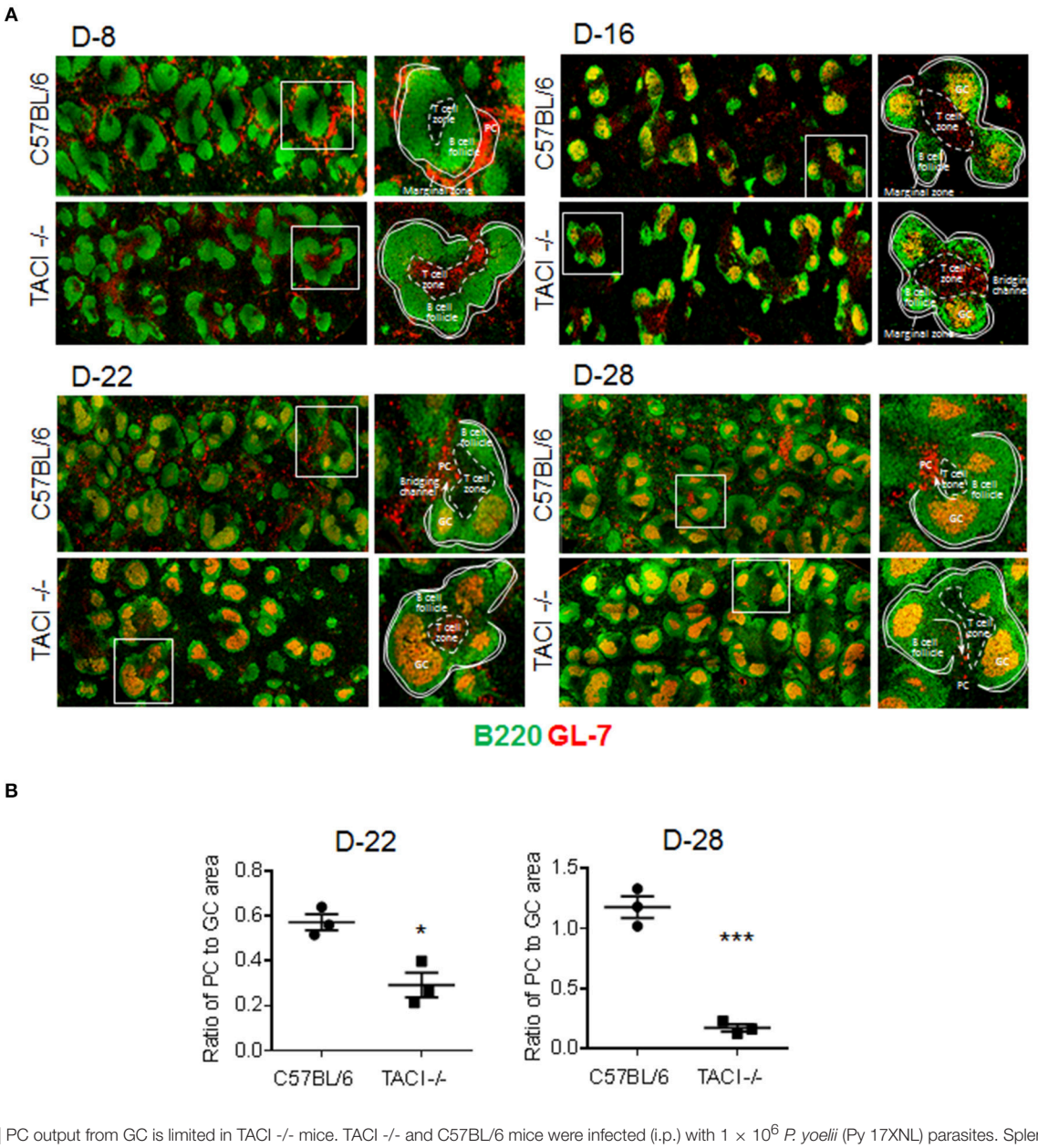

FIGURE 3 | PC output from GC is limited in TACI -/- mice. TACI -/- and C57BL/6 mice were infected (i.p.) with 1×10^6 *P. yoelii* (Py 17XNL) parasites. Splenic GC and PC were examined by microscopy on indicated days after infection. **(A)** Histologic sections of spleens were stained with anti-B220 (green) and anti-GL-7 (red) antibodies to visualize B-cell follicles (green), GC ($B220^{+}GL\text{-}7^{+}$, yellow), and PC ($B220^{low}GL\text{-}7^{+}$, orange). T-cell independent PC are located in the marginal zone and T-cell dependent PC in red pulp of the spleen, adjacent to the "Bridging channels." T cell zones were defined as central location where tightly packed follicles are distributed around and the activated T cells ($B220^{-}GL\text{-}7^{+}$, red) are located. Arrows indicate the plasmablast pathway to extrafollicular areas through the "Bridging channels." **(B)** PC output was calculated by ratio of PC foci to GCs in half spleen. Each dot represents one mouse. Magnification is 10x. $^{*}p < 0.05$ and $^{***}p < 0.001$ for TACI -/- vs. C57BL/6 comparison. Experiment was performed once.

well as activated (GL-7$^+$) cells occupying the T cell zone on day 16. By day 22, activated T cells were relocated to GC in the wild-type mice leaving the T cell-zones empty of GL-7$^+$ T cells. Also on day 22, B220^{low}GL-7$^+$ PC began exiting GC from the "Bridging channels" (36) in wild-type mice (**Figures 3A,B**). In TACI -/- mice however, not only the population of "Bridging channels" by B220^{low}GL-7$^+$ PC was sparse on day 22, but also the T cell zone was still occupied with activated GL-7$^+$ T cells in addition to the persistence of GCs in the follicules (**Figures 3A,B**). By day 28, activated T cells in the T cell zones were not present in TACI -/- spleens any longer. On the same day, the B220^{low}GL-7$^+$ PC in the wild-type mice "Bridging channels" were more abundant and remained significantly more than that of TACI -/- mice (**Figures 3A,B**). In addition to the analysis of spleens in microscopy, we also assessed the frequency of splenic IgD$^-$CD138$^+$ PC in *P. yoelii* infected mice in flow cytometry. Supporting the microscopy results, we found that the frequencies of PC on days 15 and 23 were significantly lower in TACI -/- mice than wild-type mice (**Figure 4**).

Despite a Delay in PC Response, TACI -/- Mice Develop Parasite Specific ASC and High Affinity Antibodies Following the Clearance of Infection

The discovery of significantly lower numbers of PC in TACI -/- mice as compared to wild-type mice at the time of parasite clearance (day 25), prompted us to measure *Plasmodium*-specific ASC during and after the infection. Reflecting the difference in anti-*P. yoelii* antibody levels between the two strains, we found significantly lower numbers of ASC recognizing the rMSP-1$_{19}$ protein in the spleen and bone marrow of TACI -/- mice 2 weeks post-infection (**Figure 5**). At the 2-month time point, TACI -/- mouse cells were still less than the wild-type cells but only the bone marrow ASC were statistically significantly lower in TACI -/- mice. In addition to aiding in the expansion of antibody secreting GC B cells, T$_{FH}$ cells also promote antibody affinity maturation. At 71 days post-infection, we found no difference in the avidity of antibodies directed against rMSP-1$_{19}$ protein between the mouse strains (**Supplementary Figure 4**). These observations suggested that anti-*P. yoelii* antibodies secreted from splenic ASC may be responsible for the resolution of infection in TACI -/- mice.

TACI -/- Mice Were Protected From Re-infection With *P. yoelii* Parasites

B cells are crucial for the development of protective immunity against the erythrocytic stages of *Plasmodium* (37, 38). Since TACI -/- mice eventually cleared the infection and elicited parasite specific ASC and antibodies with affinities comparable to those of wild-type mice, we assessed whether they were resistant to a second challenge as has been shown for wild-type mice (39). TACI -/- and C57BL/6 mice were infected a second time with 1 $\times$ 10^6 *P. yoelii* 11 months after the clearance of the first infection. Naïve mice were also infected as a control. As expected, higher and delayed peak parasitemia, as well as delayed parasitemia resolution (day 29 post-infection) was observed in 1X infected

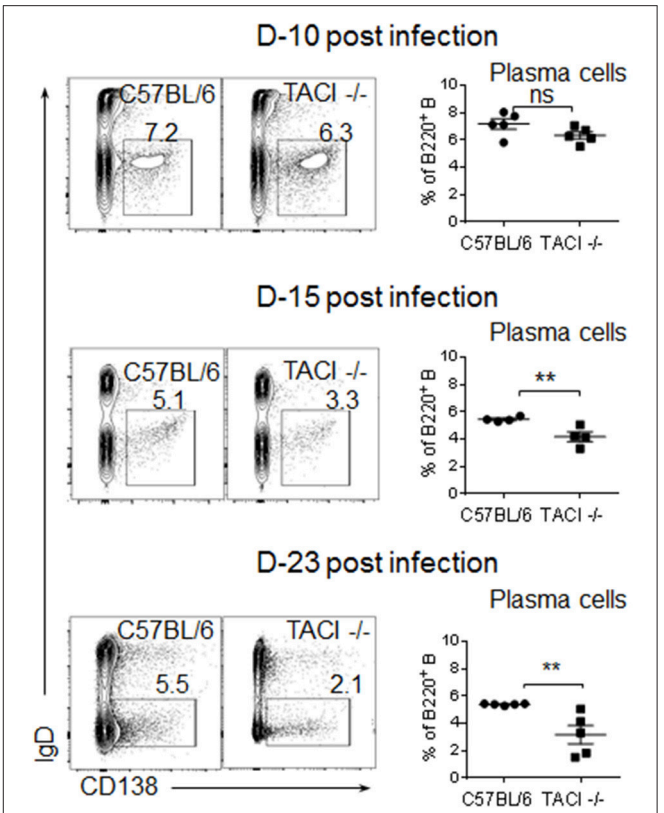

FIGURE 4 | Development of PC in infected TACI -/- mice. TACI -/- and C57BL/6 mice were infected (i.p.) with 1 $\times$ 10^6 *P. yoelii* (Py 17XNL strain) parasites. Representative dot blots of splenic B220 gated IgD-CD138$^+$ plasma cells on indicated time points are shown. Mean frequencies of PC as percentages of B cells are plotted for each time point. Each data point represents a mouse. Unpaired Student's *t*-test is used for statistical evaluation. Results are expressed as mean $\pm$ SEM ($n = 5$) from one representative experiment out of two with similar results. $^{**}p < 0.01$ indicates TACI -/- vs. C57BL/6 mice. ns, not significant.

TACI -/- mice as compared to wild-type mice (**Figure 6A**). Also, naïve wild-type mice parasitemia peaked on day 11 post-infection and was cleared by day 18. TACI -/- and C57BL/6 mice that had been infected previously were protected from a second *P. yoelii* infection, indicating a sustained long-term immunity. In these experimental groups, parasitemia levels at day 4 post-infection were 0.025% and 0.023% for TACI -/- and C57BL/6 mice, respectively, while no parasites were detected at day 11-time point.

ADOPTIVE TRANSFER OF *P. YOELII* IMMUNE B-CELLS AFFORD PROTECTION

The association between antibody development and parasite clearance in both the strains suggested that the resistance to a second infection is likely a result of the persistence of B cell memory and/or PC. In a cerebral malaria model, CD19$^+$ B cells from *Plasmodium berghei* recovered mice confers protection in naïve mice (40). To assess the role of parasite experienced B cells

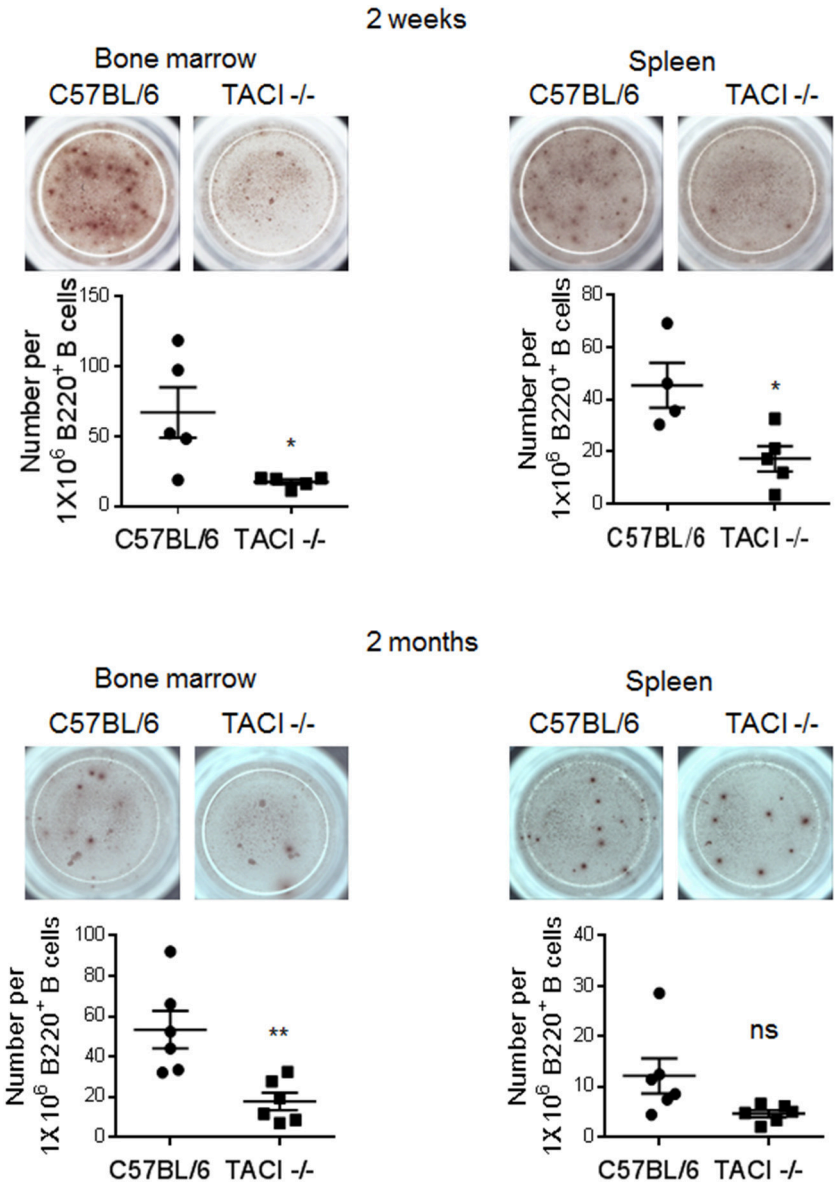

FIGURE 5 | TACI deficiency causes delayed generation of rMSP-1_{19}-specific ASC. Spleens and bone marrow cells were harvested 2 weeks and 2 months post-infection to examine the frequency of antigen-specific IgG antibody secreting cells (ASC) in ELISPOT assay. The number of rMSP-1_{19}-specific ASC per 1×10^6 $B220^+$ B cells were quantified and plotted. Each data point represents one mouse. Unpaired Student's *t*-test is used for statistical evaluation. Results are expressed as mean ± SEM ($n = 4$–6) from one representative experiment out of two with similar results. *$p < 0.05$ and **$p < 0.01$ indicate TACI -/- vs. C57BL/6 mice. ns, not significant.

in the protection of TACI -/- mice against *P. yoelii* infection, we performed adoptive transfer experiments. Naïve B cells from C57BL/6 mice or B cells from previously infected C57BL/6 and TACI -/- mice (4 months earlier) were used as donor cells. Thirty million B cells were adoptively transferred into each naïve C57BL/6 mouse two hours before the experimental challenge with 1×10^6 *P. yoelii* parasites. In mice receiving naïve B cells, peak parasitemia level and time-point (day 13) (**Figure 6B**) were comparable to those of naïve mice receiving *P. yoelii* for the first time (**Figure 1A**). In contrast, C57BL/6 mice that were adoptively transferred with *P. yoelii* experienced cells from either strain had very low peak parasitemia levels (day 6), which resolved by day 10 post-infection in both mouse strains (**Figure 6B**). Thus, the B cell immunity developed after *P. yoelii* infection of TACI -/- mice is transferable and sufficient to render naïve mice resistant to *P. yoelii* challenge.

DISCUSSION

Infant susceptibility to malaria is likely compounded by the unique features of their immune system. Both the qualitative and quantitative antibody responses to malaria

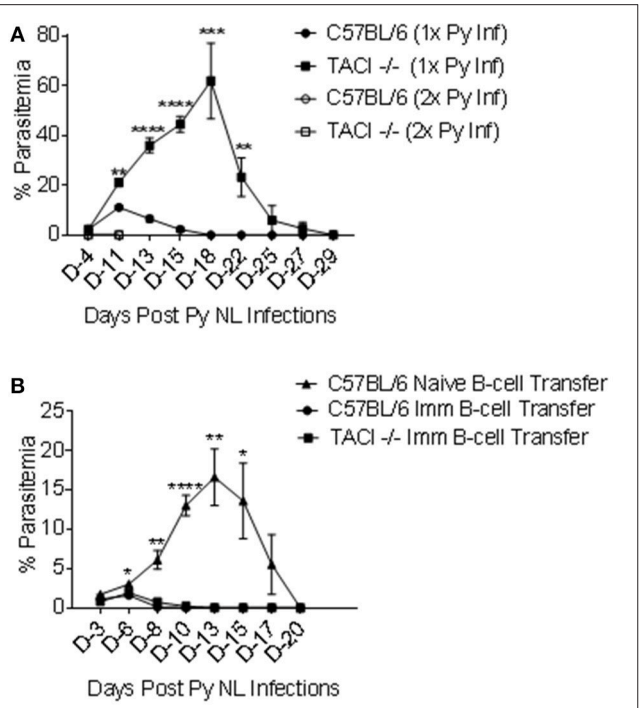

FIGURE 6 | TACI -/- mice are resistant to re-infection with *P. yoelii* and *P. yoelii* experienced TACI -/- mice B cells are protective. **(A)** TACI -/- and C57BL/6 mice were re-infected (i.p.) with 1×10^6 *P. yoelii* (Py 17XNL) parasites 11 months after the first infection (2x Py inf). A second group of naïve TACI -/- and C57BL/6 mice was infected for the first time to recapture the first infection parasitemia (1x Py inf). Parasitemia levels were evaluated by blood smears at indicated days post challenges. Results are expressed as mean % parasitemia ± SEM for 10 mice per group for first challenge, and for 5 mice per group for the second challenge. Unpaired student's *t*-test was used for statistical evaluation. *indicates $p < 0.05$, **$p < 0.01$;***$p < 0.001$; and ****$p < 0.0001$ when comparing 1x Py NL and 2x Py infected TACI -/- mice. Experiment was performed once. **(B)** B cells from naive C57BL/6 or *P. yoelii* 17XNL infected C57BL/6 and TACI -/- mice were adoptively transferred into 3 to 4 naïve C57BL/6 mice. Two hours after B-cell transfer, mice were challenged (i.p.) with 1×10^6 *P. yoelii* parasites and parasitemia were assessed on indicated days. Unpaired student's *t* test was used for statistical evaluation. Results are expressed as mean % parasitemia ± SEM. *indicates $p < 0.05$, **$p < 0.01$;***$p < 0.001$; and ****$p < 0.0001$ when comparing the naïve B cell transfer and the TACI -/- B cell transfer groups. Experiment was performed once.

are tightly associated with the age of infected infants (41–43). Incomplete understanding of the underlying molecular and cellular requirements supporting the development and maintenance of long lasting anti-malarial memory B cell and PC responses in infants hamper the development of effective malaria vaccines in children. In this study, we investigated the role of TACI in resistance to malaria infection because TACI is important for the development and maintenance of PC (5, 8–10, 15).

Both human and mouse studies suggested a role for the TACI ligand BAFF in host response to malaria infection. Elevated serum BAFF has been measured in adults experimentally infected with *P. falciparum* and in children with acute malaria (13, 14). The increase in serum BAFF levels is accompanied by a decrease in BAFF-R and an increase in TACI and BCMA on B cells of malaria-infected children (13). Despite these reports, the contribution of TACI in malaria infection has not been explored. We have previously shown that low TACI expression in neonatal B cells is responsible for the inability of this age group to respond to polysaccharide vaccines (15). We hypothesized that low TACI expression might also promote the suboptimal anti-malarial humoral immune response observed in infants. Significantly elevated levels and the delayed resolution of parasitemia in *P. yoelii* infected TACI -/- mice indicated that TACI is important for controlling malaria. Elicited protective humoral immunity was likely responsible for the clearance of *Plasmodium* in TACI -/- mice because the delayed clearance of *P. yoelii* coincided with the late emergence of anti-malaria antibodies, and B cells transferred from *P. yoelii* immune TACI -/- mice afforded protection in naïve wild-type mice, and TACI -/- mice rechallenged with *P. yoelii* resisted infection. Moreover, recovered TACI -/- mouse spleens contained *P. yoelii* specific ASC with comparable antibody avidity to those secreted from wild-type mice. Both T-cell dependent and independent mechanisms are involved in the development of anti-malaria antibodies (30). Since TACI expression is required for the development of T cell independent antibody responses, impairment in the antibodies targeting malarial T cell independent antigens likely have contributed to delayed parasitemia resolution. Indeed, we detected the appearance of $GL7^+$ PC in the splenic marginal zones of wild-type mice as early as day 8 after infection while the TACI -/- mice marginal zone PC response was absent. Additional association between TACI expression and TI B cell responses to malaria may be related to the effect of toll-like receptor (TLR) 9 on TACI expression. We have previously shown that the TLR9 agonist CpG boosts BAFF and APRIL mediated PC generation by strongly upregulating the expression of TACI on B cells (44). Since, TLR9 deficiency compromises host control of parasitemia (45), TACI deficiency may also be negating the beneficial effect mediated by malaria TLR9 agonists (46).

Previous reports have shown that the formation of T_{FH} and GC is amplified in TACI -/- mice after immunization (11) and infection (12). However, while the increased T_{FH} and GC development was beneficial in eliciting high affinity antibodies that helped resist *C. rodentium* infection (12), nitrophenyl-chicken gamma globulin immunized TACI -/- mice generated lower levels of nitrophenyl-specific antibodies with diminished affinities (11). The immunization study also demonstrated an ablated ASC development in TACI -/- mice, which was attributed to the decrease in PC survival, a likely consequence of the absence of TACI mediated survival signals (11). Diminished ASC maintenance was reported in another study where influenza infected TACI -/- mice developed lower levels of virus-specific ASC compared to wild-type mice (10). Like the previous reports (11, 12), we observed exaggerated magnitude and kinetics in TACI -/- mice T_{FH} and GC formation after *Plasmodium* infection. Despite the augmented T_{FH} and GC B responses, TACI -/- mice were not able to control the parasitemia partly due to the delay in the emergence of PC from GC and the development of parasite specific ASC. Although the increased expression

of ICOSL may be contributing to the persistence of Tfh and GC B cells, it can also be a result of persistent stimulation with parasite antigens as has been shown in a model where boosting with peptide stimulates T_{FH} response without increasing B cell response (47).

Supporting the clinical observations where high level of BAFF is measured in *Plasmodium* infected individuals (13, 14), stimulation of monocytes with soluble *Plasmodium* molecules, soluble schizont fraction of Plasmodium falciparum antigen (sPFAg) and hemozoin (HZ) both induce the expression of BAFF (48). Although these studies suggested a possible involvement of increased serum BAFF in the activation of B cells and generation of antibody secreting cells in malaria, by analyzing *P. yoelii* infected mice, Liu et al detected a decrease in the number of dendritic cells (DCs) expressing membrane BAFF after malaria challenge (49). Since multimeric BAFF, but not trimeric serum BAFF, is able to promote PC by engaging TACI (50), the authors proposed a possible link between the disappearance of malaria specific ASC with the decrease in BAFF expressing DCs in malaria endemic regions. It remains to be seen whether the decrease in BAFF expressing DCs is accompanied by a decrease in the other TACI ligand, APRIL, and if not, whether APRIL can or cannot compensate for the diminished BAFF expressing DCs in sustaining the survival of ASC. Regardless of the significance of the changes in BAFF and APRIL expression, our study highlights the importance of TACI mediated development of ASC in controlling malaria infection. Interestingly, despite the delay in the generation of malaria specific antibodies and recovery from infection, TACI deficient mice remained resistant to second malaria challenge even after 11 months. Malaria specific B cells elicited in TACI -/- mice were not only able to clear the infection but also could render naïve mice resistant to malaria challenge.

Our study highlights the importance of TACI mediated control of T_{FH} and GC response, and ASC development during malaria infection. These findings may have implications in understanding the immunobiological bases of infant-susceptibility to malaria since TACI expression is severely reduced in neonatal B cells in mice and in humans (15, 51). Moreover, as in TACI -/- mice, the development of humoral immune response is delayed in children from malaria endemic area (4, 52). Further work is needed in infant populations and in murine models of neonatal malaria to elucidate the impact of low TACI expression on the phenotype of T_{FH} cells during malaria infection and vaccine response.

AUTHOR CONTRIBUTIONS

MA, MP, and JY: designed the study and wrote the manuscript; MP, JY, MW, SD, AY, TS, and BS: performed the experiments; MP, JY, MW, SD, AY, TS, BS, AM, and MA: analyzed data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02612/full#supplementary-material

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ORIGINAL RESEARCH
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Plasmodium chabaudi AS Infection Induces CD4$^+$ Th1 Cells and Foxp3$^+$T-bet$^+$ Regulatory T Cells That Express CXCR3 and Migrate to CXCR3 Ligands

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Control and elimination of blood-stage *Plasmodium chabaudi* AS infection requires CD4$^+$ Th1 cells that secrete IFN-γ and T follicular help (Tfh) cells together with B cell production of antibody. Foxp3$^+$ regulatory T cells (Tregs) are also crucial to protect the host from immunopathology and severe disease, but these cells can suppress protective immune responses to malaria. The chemokine receptor CXCR3 expressed by activated T cells is important for trafficking of CD4$^+$ Th1 cells to sites of inflammation and infection. Previous studies demonstrated CXCR3 is expressed on CD4$^+$ T cells in the spleen during malaria, but the phenotype was not defined. We identified the phenotype of CD4$^+$ T cells that expressed CXCR3 in C57BL/6 (B6) mice during acute *P. chabaudi* AS infection by analyzing expression of the transcription factors T-bet and Foxp3. We also investigated if CXCR3 contributes to control of parasite replication and survival. The frequency and number of CD4$^+$CXCR3$^+$ T cells increased dramatically in the spleen of infected B6 mice coincident with increased CD4$^+$IFN-γ$^+$ T cells. CXCR3 was up-regulated on effector CD4$^+$Foxp3$^-$ T cells as well as Foxp3$^+$ Tregs. Consistent with our previous observations, CD4$^+$T-bet$^+$Foxp3$^-$ T cells increased in B6 mice during acute infection. T-bet$^+$Foxp3$^+$ Tregs also increased significantly and a high frequency of these cells expressed CXCR3 supporting the notion that these cells may be Th1-like Tregs. Despite this, the percentage of CD4$^+$Foxp3$^+$ Tregs from infected B6 mice that migrated *in vitro* to the CXCR3 ligands CXCL9 and CXCL10 was significantly less than naïve mice. To investigate the *in vivo* contribution of CXCR3 to control of acute blood-stage malaria, we compared the course and outcome of *P. chabaudi* AS infection in wild-type (WT) B6 and CXCR3-deficient mice. Parasitemia levels were significantly higher around the time of peak parasitemia in CXCR3$^{-/-}$ compared to WT mice but survival was similar suggesting a role for CXCR3 in controlling parasite replication during acute *P. chabaudi* AS infection. Together, our

findings indicate Th1-like CD4$^+$T-bet$^+$Foxp3$^+$ Tregs that express CXCR3 are induced during acute blood-stage malaria and suggest CXCR3 expression on CD4$^+$ Th1 cells may contribute to their migration to the spleen.

Keywords: malaria, CXCR3, CD4$^+$ T cells, regulatory T cells, T-bet

INTRODUCTION

Malaria remains a major global health threat with 90% of the disease burden in sub-Saharan Africa (1). In 2016, there were 216 million cases of malaria world-wide which represents an increase of 5 million cases compared to 2015 even though the number of deaths remained at ∼445,000 per year. Despite extensive studies in humans and mice infected with *Plasmodium* to identify the immune mechanisms required for protection against blood-stage infection, important gaps in our knowledge remain. CD4$^+$ T cell-B cell interactions are essential for control of parasite replication and elimination of infection (2). CD4$^+$ Th1 cells that express T-bet and secrete IFN-γ and T follicular helper (Tfh) cells, crucial for generating antibody-mediated immunity, play important roles (2). Immunoregulatory mechanisms including the anti-inflammatory cytokine IL-10 and regulatory T cells (Tregs) are vital to protect the host from immunopathology and severe disease (3–5). On the other hand, such mechanisms may suppress protective immune responses.

Although earlier studies on the role of CD4$^+$Foxp3$^+$ Tregs in immunity to malaria were not conclusive, recent findings in mice infected with *Plasmodium chabaudi* AS or *P. yoelii* support the notion that Tregs suppress Th1 as well as Tfh cell responses (4, 6, 7). Indeed, higher blood parasitemia levels are associated with higher frequencies of CD4$^+$Foxp3$^+$ Tregs in humans and mice with malaria. Conversely, a lower frequency of Tregs is associated with better disease outcome. During the acute phase of *P. chabaudi* AS, C57BL/6 (B6) mice, used in the present study, have a significant increase in effector CD4$^+$ Th1 cells that express T-bet and secrete IFN-γ (6, 8). The accumulation and expansion of CD4$^+$ Th1 cells that secrete IFN-γ in the spleen are essential for control and elimination of *P. chabaudi* AS infection (9). Although CD4$^+$Foxp3$^+$ Tregs increase significantly in infected compared to naïve B6 mice, there is a high ratio of effector CD4$^+$ T cells to Tregs in these hosts during acute *P. chabaudi* AS infection (6).

The lymphocyte-specific chemokine receptor CXCR3 expressed by activated T cells as well as NK cells is important for CD4$^+$ Th1 cell migration to sites of inflammation and infection (10, 11). Interaction of CXCR3 with its ligands, the C-X-C chemokines CXCL9 (monokine induced by IFN-γ), CXCL10 (interferon-induced protein-10), and CXCL11 (interferon-inducible T-cell alpha chemoattractant) contributes to Th1 cell differentiation (12). The C-X-C chemokines are induced by IFN-γ and are produced by several immune cells including macrophages and dendritic cells as well as non-immune cells.

CXCR3 and other chemokine receptors have been demonstrated to be up-regulated in CM patients and during ECM in mice (13–16). Susceptibility to ECM in *P. berghei* ANKA-infected B6 mice requires CXCR3 expression on pathogenic CD8$^+$ T cells (17). *Cxcr3*$^{-/-}$ mice are protected from ECM due to reduced CD8$^+$ T cell sequestration in the brain. Interestingly, CXCR3 expression is also highly up-regulated on CD4$^+$ and CD8$^+$ T cells in the spleen during *P. berghei* ANKA infection (15). However, neither the role of CXCR3 expression on T cells in the spleen during malaria nor the phenotype of the T cells, especially of CD4$^+$ T cells, expressing this chemokine receptor have been investigated in mice infected with *P. berghei* ANKA or in other rodent malaria models. In the present study, we examined CXCR3 expression on CD4$^+$ T cells in the spleen of B6 mice infected with *P. chabaudi* AS and identified the CD4$^+$ T cell population that express it.

The T-box transcription factor T-bet is essential for Th1 cell differentiation and effector function due to its ability to activate transcription of the IFN-γ gene (18). Interestingly, T-bet is expressed on a subset of CD4$^+$Foxp3$^+$ T cells and is thought to be involved in the homeostasis and function of these cells during Th1 inflammation (19). Recently, it was observed that Foxp3$^+$ Tregs that express T-bet increase in individuals with acute *P. vivax* infection (20). In addition to activating transcription of the IFN-γ gene, T-bet induces the transcription of other Th1-associated genes including *Cxcr3* inducing up-regulation of CXCR3 expression on effector CD4$^+$ T cells (21). Increased CXCR3 expression on CD4$^+$Foxp3$^+$ T cells likewise requires T-bet and occurs via a IFN-γ-dependent mechanism (19).

Given the complex interactions between CXCR3 and the transcription factor T-bet on effector CD4$^+$Foxp3$^-$ T cells and CD4$^+$Foxp3$^+$ Tregs, we questioned if these Th1-associated markers are differentially expressed on CD4$^+$ T cell populations during acute *P. chabaudi* AS infection in B6 mice. To address this, we investigated if CXCR3 expression is up-regulated on CD4$^+$ T cell populations in the spleen of infected mice. We also determined if CXCR3 and T-bet are co-expressed on effector CD4$^+$Foxp3$^-$ T cells and CD4$^+$Foxp3$^+$ Tregs during infection. Because we observed that C-X-C chemokine expression is up-regulated in the spleen during *P. chabaudi* AS infection, we performed *in vitro* migration assays to study the chemotactic ability of CD4$^+$ T cells from infected mice to CXCL9, CXCL10, and CXCL11. Finally, we investigated the *in vivo* role of CXCR3 to immunity to blood-stage malaria by determining the course and outcome of *P. chabaudi* AS infection in wild-type (WT) B6 compared to CXCR3-deficient mice.

MATERIALS AND METHODS

Mice and Parasites

WT B6 mice were purchased from Charles River Laboratories (St. Constant, QC). B6.129P2-Cxcr3^{tm1Dgen}/J (CXCR3$^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor,

ME). Mice were maintained in the animal facility of the Research Institute of the McGill University Health Center (Montreal, QC) under specific-pathogen-free conditions. Female mice, 6–8 weeks old, were used for all experiments. Experiments were conducted using procedures approved by the Canadian Council on Animal Care and the Animal Care Committee of the Research Institute of the McGill University Health Center. Blood-stage *P. chabaudi* AS parasite was maintained in mice by weekly passage as previously described (22). Mice were infected by intraperitoneal (i.p.) injection of 1×10^6 parasitized red blood cells (pRBC). Parasitemia was monitored in the blood by microscopic examination of Diff-Quick (Fisher Scientific)-stained blood smears.

Cell Preparation

Spleens from naïve and *P. chabaudi* AS infected B6 mice were collected aseptically at the indicated times post-infection (p.i.). To prepare single cell suspensions, tissues were perfused with PBS containing 1% FCS (HyClone Laboratories, Logan, UT), teased apart, and gently pressed through a sterile fine wire mesh. The cells were centrifuged, re-suspended in 0.175 M NH_4Cl to lyse red blood cells (Sigma-Aldrich, St. Louis, MO), washed, and re-suspended in complete RPMI 1640 medium (Life Technologies, Burlington, ON, Canada) supplemented with 5% heat-inactivated FCS, 25 mM HEPES (Life Technologies), 0.12% gentamicin (Life Technologies), and 2 mM glutamine (Life Technologies). The total number of spleen cells obtained from individual mice was determined using a hemocytometer. Cell viability was determined by trypan blue exclusion (Life Technologies) and was always >95%. For some experiments, $CD4^+$ T cells were purified from single cell suspensions prepared from spleens of naïve and infected mice at the indicated times p.i. using a negative $CD4^+$ T cell isolation kit (Miltenyi Biotec) following the manufacturer's instructions.

Immunophenotyping

Single cell suspensions of splenocytes, prepared as described above, were adjusted to $1–2 \times 10^6$ cells/ml and stained with viability dye (eFluor® 780 or eFluor® 506; eBioscience, San Diego, CA). Prior to immunophenotyping, Fc receptors were blocked with anti-mouse CD16/CD32 monoclonal antibody (mAb) (clone 2.4G2; BD Biosciences, San Jose, CA) and the cells were surface stained with FITC-conjugated anti-CD4 mAb (clone GK1.5; eBioscience) and PE-conjugated anti-CXCR3 mAb (clone CXCR3-173; eBioscience). For intracellular staining, the cells were surface stained, fixed, and permeabilised using an intracellular fixation kit (eBioscience) according to the manufacturer's instructions, and intracellularly stained with APC-labeled anti-Foxp3 (clone FJK-16s; eBioscience) and anti-T-bet (clone 4B10; eBioscience) mAb. For each antibody, staining was compared to cells stained with an appropriate isotype control antibody. Flow cytometry was performed using a FACSCanto (BD Biosciences) for acquisition, and data were analyzed using FlowJo software (TreeStar).

Intracellular Cytokine Staining

Purified $CD4^+$ T cells obtained from naïve and infected mice were resuspended to 1×10^6 cells/ml in complete RPMI 1640 medium and stimulated with 50 ng/ml PMA and 1 μM ionomycin (Sigma-Aldrich) for 5 h at 37°C and 5% CO_2 in the presence of 1 μl/ml Brefeldin A (BD Biosciences) to inhibit cytokine secretion. Cells were harvested and surface stained with fluorochrome-conjugated antibodies as described above. After fixation and permeabilization, the cells were stained for intracellular cytokine expression using APC-conjugated, anti-IFN-γ mAb (clone XMG1.2, eBioscience). Cells were also stained for intracellular T-bet expression as described above to identify Th1 cells. Cells were gated on $CD4^+$ cells for FACS analysis and data were analyzed using FlowJo software (TreeStar).

Chemotaxis Assay

For migration studies, 5×10^5 purified $CD4^+$ T cells in complete RPMI 1640 medium were added to the top chambers of a 96 well Transwell plate with a pore size of 5 μm (Corning Costar, Acton, MA). Mouse recombinant chemokines CXCL9, CXCL10, CXCL11, and CCL21 (Peprotech, Rocky Hill, NJ) were diluted in complete RPMI 1640 medium and added to the bottom chamber of the Transwell plate to a final concentration of 200 μM for CXCL9 and CXCL11 and 100 μM for CXCL10 and CCL21. As a control, complete RPMI 1640 medium alone was added to the bottom chamber. After incubation for 4 h at 37°C in 5% CO_2, the cells were collected from the bottom chamber and counted in a hemocytometer. The chemotaxis index was calculated by dividing the number of cells that migrated in response to chemokines by the number of cells in the bottom chamber containing medium alone.

To identify the population of cells that had migrated to each chemokine, cells were collected from individual bottom chambers and surface stained with FITC-conjugated CD4 mAb followed by intracellular staining with APC-conjugated Foxp3 mAb as previously described. Cells were analyzed by FACS and the percentage of $CD4^+Foxp3^+$ Tregs in the migrated cell population was determined.

RT-PCR

To investigate the expression of chemokine genes, spleens were harvested from naïve and infected B6 mice on the indicated days p.i. and immediately snap-frozen in liquid nitrogen. Frozen tissues were stored at −80°C until processing. Total RNA was extracted and purified using the RT^2-qPCR-grade mRNA isolation kit (Qiagen) according to the manufacturer's instructions. RNA integrity was verified by denaturing agarose gel electrophoresis, followed by staining with ethidium bromide, and visualized on a UV trans-illuminator. Purity and quantification of RNA were determined by UV absorbance ratio of A260/A280. For first strand cDNA synthesis, 1 μg of purified RNA was used. First strand cDNA synthesis and RT-PCR were performed using reagents and protocols provided by the manufacturer in the RT^2 Profiler® PCR Kit for Mouse Chemokines and Chemokine Receptors (Qiagen). Reactions were performed in a StepOne® real-time PCR instrument (Applied Biosystems) using a pre-set, standard run thermal cycling condition of 40 cycles. Raw C_t data

were analyzed using the RT^2 Profiler® software provided on the manufacturer's web portal. Data are presented as fold increase in mRNA of infected over naïve mice.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). The statistical significance of differences between groups was analyzed using Kruskal Wallis for one-way analysis of variance. Survival data were analyzed using Martel-Cox log rank test. All statistical analyses were performed using Prism 5 software (GraphPad, San Diego, CA). $p < 0.05$ was considered significant.

RESULTS

CXCR3 Expression Increases on Splenic CD4$^+$ T Cells During Acute *P. chabaudi* AS Infection and Is Expressed on Both Effector CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ Regulatory T Cells

Previous studies showed that CXCR3 expression increases on CD4$^+$ and CD8$^+$ T cells in the spleen during *P. berghei* ANKA infection suggesting this chemokine receptor may be important in T cell trafficking to the spleen during malaria (15). During *P. chabaudi* AS infection, the spleen is the major site of accumulation of CD4$^+$ T cells especially CD4$^+$T-bet$^+$IFN-γ^+ Th1 cells that are essential to control parasitemia during the first 2 weeks after infection (6, 23). To further investigate the role of CXCR3 in *P. chabaudi* AS infection, we determined CXCR3 expression on CD4$^+$ T cells in the spleen of infected B6 mice using the gating strategy shown in **Supplementary Figure 1A**. The frequencies and numbers of CD4$^+$CXCR3$^+$ T cells increased significantly in the spleen of *P chabaudi* AS-infected compared to naïve B6 mice on days 8 and 11 p.i. (**Figures 1A,B**). Importantly, the increases in CD4$^+$CXCR3$^+$ T cells coincided with significant increases in CD4$^+$IFN-γ^+ T cells suggesting the importance of CXCR3 in the accumulation of effector Th1 cells in the spleen during acute *P. chabaudi AS* infection (**Figure 1C**).

Previously, we observed that CD4$^+$Foxp3$^+$ Tregs increase significantly in the spleen of infected B6 mice during acute *P. chabaudi* AS infection followed by a significant increase in effector CD4$^+$T-bet$^+$IFN-γ^+ Th1 cells around the time of peak parasitemia (6). Immunohistochemical staining revealed that CD4$^+$Foxp3$^+$ Tregs are located almost exclusively in the T cell areas of the white pulp within 6 days p.i. and are still evident on day 10 p.i. To address if CXCR3 is expressed differentially on CD4$^+$ T cell populations during blood-stage malaria, we examined CXCR3 expression on CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ T cells (**Supplementary Figure 1A**). A similarly low frequency of CD4$^+$ T cells from naïve B6 mice expressed CXCR3 in gated CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ T cells (**Figure 1D**). The frequencies of cells expressing CXCR3 increased among effector CD4$^+$Foxp3$^-$ T cells as well as CD4$^+$Foxp3$^+$ Tregs as the infection progressed, with ∼50% of effector CD4$^+$Foxp3$^-$ T cells expressing CXCR3 compared to 33% of CD4$^+$Foxp3$^+$ Tregs on day 8 p.i. The level of CXCR3 expression was up-regulated on CD4$^+$Foxp3$^+$ T cells from infected compared to naïve B6 mice as shown by significantly higher MFIs on days 8 and 11 p.i. (**Supplementary Figure 1B**). Although there were significant increases in the frequencies and numbers of CD4$^+$Foxp3$^-$ as well as CD4$^+$Foxp3$^+$ T cells expressing CXCR3 on days 8 and 11 p.i. compared to naïve B6 mice, the increases were significantly greater in effector CD4$^+$ T cells compared to CD4$^+$Foxp3$^+$ Tregs (**Figures 1E,F**).

T-bet and CXCR3 Expression Are Up-Regulated on Effector CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ Regulatory T Cells During Blood-Stage *P. chabaudi* AS

Consistent with our previous observations, CD4$^+$Foxp3$^-$ T cells expressing T-bet increased dramatically in the spleen of infected B6 mice on days 8 and 11 p.i. (**Figure 2A**) (6). The frequency and number of CD4$^+$Foxp3$^+$ cells expressing T-bet also increased during infection albeit to lower levels than effector CD4$^+$ T cells expressing T-bet. On days 8 and 11 p.i., the frequencies and numbers of CD4$^+$T-bet$^+$Foxp3$^-$ T cells were significantly higher in infected compared to naïve B6 mice, while significant increases in frequency and number of CD4$^+$T-bet$^+$Foxp3$^+$ cells were evident only on day 11 p.i. (**Figures 2B,C**). Notably, the frequency and number of CD4$^+$T-bet$^+$Foxp3$^+$ cells were significantly less than the frequency and number of CD4$^+$T-bet$^+$Foxp3$^-$ T cells on days 8 and 11 p.i.

In addition to activating transcription of the IFN-γ gene, T-bet also induces the transcription of other Th1-associated genes including *Cxcr3* and increases cell surface CXCR3 expression on effector CD4$^+$ T cells (18). Up-regulation of CXCR3 expression on CD4$^+$Foxp3$^+$ T cells also requires T-bet and occurs via an IFN-γ-dependent mechanism (19). To understand the relationship between T-bet and CXCR3 expression during blood-stage malaria, we determined if these markers are co-expressed by CD4$^+$ T cell populations in the spleen. CD4$^+$ T cells from naïve and infected B6 mice were surface stained for CD4 and CXCR3 followed by intracellular staining for Foxp3 and T-bet, and marker expression was analyzed in gated CD4$^+$ cells by flow cytometry. First, we analyzed CXCR3 expression on Foxp3$^-$ and Foxp3$^+$ cells in the gated T-bet$^-$ populations (**Figure 3A**). We observed small but significant increases in the frequencies of Foxp3$^-$T-bet$^-$ and Foxp3$^+$T-bet$^-$ cells that expressed CXCR3 on day 8 p.i. compared to naïve B6 mice with a further significant increase in the frequency of CXCR3$^+$ cells in the Foxp3$^-$T-bet$^-$ population on day 11 p.i. (**Figure 3B**). We then gated on the T-bet$^+$ population and analyzed CXCR3 expression within the two populations (**Figure 3A**). We observed a dramatic increase in the frequency of cells expressing CXCR3 among Foxp3$^-$T-bet$^+$ as well as Foxp3$^+$T-bet$^+$ cells on days 8 and 11 p.i. (**Figure 3C**). Together, these findings demonstrate that low frequencies of effector CD4$^+$Foxp3$^-$ T cells and CD4$^+$Foxp3$^+$ Tregs express CXCR3 in the absence of T-bet expression during *P. chabaudi* AS infection. Importantly, CXCR3 expression was highly up-regulated, regardless of Foxp3 expression, and was co-incident with up-regulated T-bet expression and increased frequency of IFN-γ^+ effector CD4$^+$ T cells on day 8 p.i. as shown above.

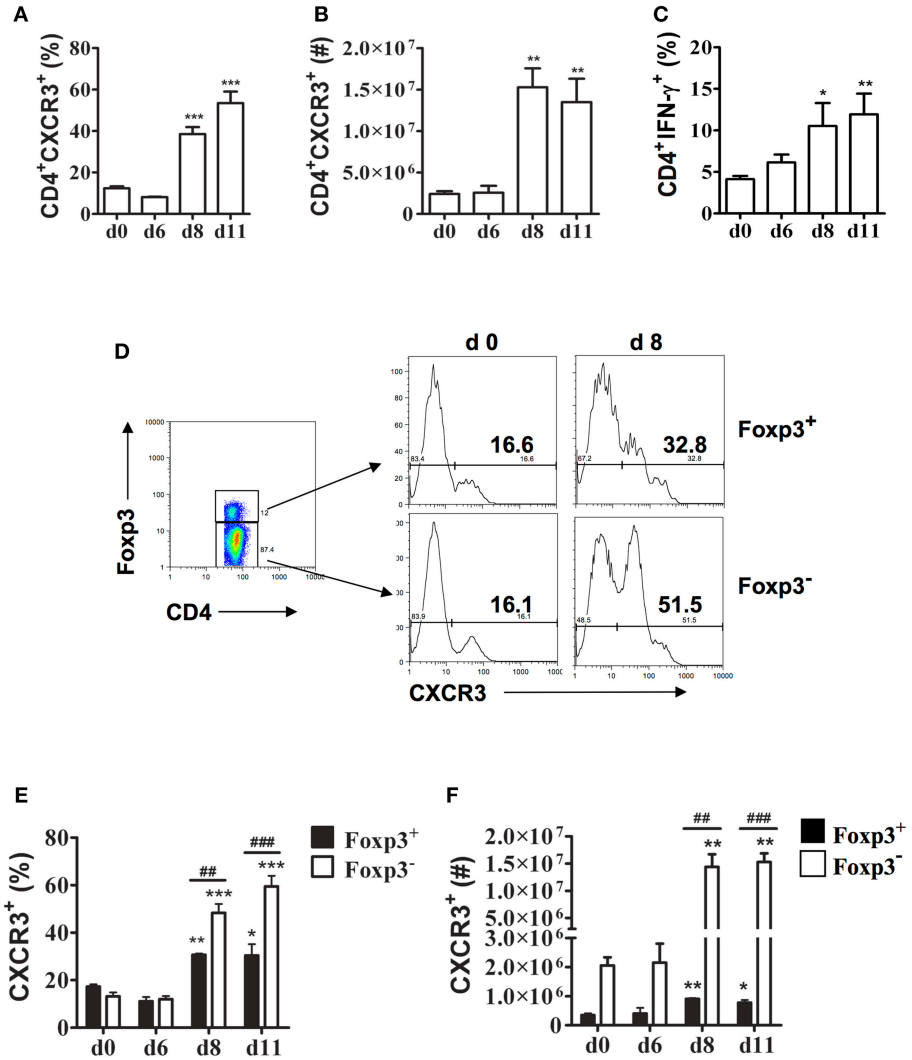

FIGURE 1 | CXCR3 expression is up-regulated on splenic $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells during *P. chabaudi* AS infection. Spleen cells were collected from naïve (d0) and infected B6 mice on the indicated days after i.p. infection with 1×10^6 *P. chabaudi* AS pRBC. **(A,B)** The cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CXCR3 mAbs and analyzed by flow cytometry. The frequency **(A)** and number **(B)** of $CD4^+CXCR3^+$ cells are shown. Data are presented as mean ± SEM and are representative of one of two replicate experiments with 3–5 mice at each time point. $^{**}p < 0.01$; $^{***}p < 0.005$. **(C)** $CD4^+$ T cells were purified from the spleens of naïve and infected B6 mice on the indicated days p.i. and stimulated with 50 ng/ml PMA and 1 μM ionomycin for 5 h in the presence of 1 μl/ml Brefeldin A. Cells were harvested and surface stained with FITC-conjugated anti-CD4 mAb, fixed and permeabilised, and intracellularly stained with APC-conjugated anti-IFN-γ-mAb. Gated $CD4^+$ cells were analyzed by flow cytometry for the frequency of IFN-γ^+ cells. Data are presented as mean ± SEM and are representative of one of two replicate experiments with 3–5 mice at each time point. $^{*}p < 0.05$; $^{**}p < 0.01$. **(D–F)** Spleen cells from naïve (d0) and infected WT mice were collected and surface stained with fluorochrome-conjugated anti-CD4 and anti-CXCR3 mAbs and intracellularly stained with APC-labeled anti-Foxp3 mAb. The gating strategy used for FACS analysis and representative plots from a naïve (d0) mouse and a mouse on day 8 p.i. are shown **(D)**. The frequency **(E)** and number **(F)** of $CXCR3^+$ cells within the $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ populations at various times p.i. are shown. Data are presented as mean ± SEM and are representative of one of two replicate experiments with 3–5 mice at each time point. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.005$ for $Foxp3^-$ and $Foxp3^+$ cells from infected compared to naïve (d0) mice. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.005$ for $Foxp3^-$ compared to $Foxp3^+$ cells.

$CD4^+$ T Cells From Infected B6 Mice Migrate *in vitro* to C-X-C Chemokines

CXCR3 and its ligands, the IFN-γ-inducible C-X-C chemokines CXCL9, CXCL10, and CXCL11, are important for migration of activated $CD4^+$ Th1 cells to inflamed tissues and sites of infection (24). To determine if CXCR3 ligands are induced during *P. chabaudi* AS infection, we examined chemokine mRNA expression in the spleen of B6 mice at various times after infection by microarray. We observed *Cxcl9* and *Cxcl10* but not *Cxcl11* expression was up-regulated in the spleen of infected B6 compared to naïve mice (**Figure 4A**).

To determine if increased expression of CXCR3 on $CD4^+$ T cells during malaria was reflected in their ability to migrate *in vitro* to C-X-C chemokines, $CD4^+$ T cells were purified from the

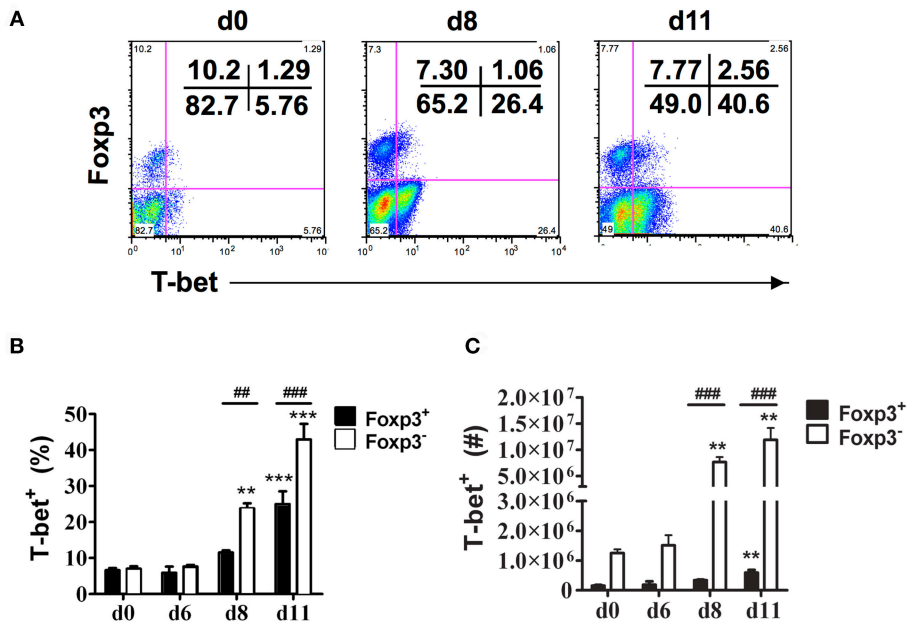

FIGURE 2 | T-bet expression is up-regulated on both effector CD4$^+$ T cells and CD4$^+$Foxp3$^+$ Tregs. Spleen cells were collected from naïve (d0) and infected B6 mice on the indicated days after i.p. infection with 1 × 10^6 *P. chabaudi* AS pRBC. The cells were surface stained with FITC-conjugated anti-CD4$^+$ mAb and intracellularly stained with fluorochrome-conjugated anti-Foxp3 and anti-T-bet mAbs. Gated CD4$^+$ T cells were analyzed by flow cytometry for expression of Foxp3 and T-bet. **(A)** Representative dot plots from a naïve (d0) mouse and a mouse on day 8 and day 11 p.i. showing the frequency of T-bet expression in the CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ populations. The frequency **(B)** and number **(C)** of T-bet$^+$ cells in the CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ populations at various times p.i. are shown. Date are presented as mean ± SEM and are representative of one of two replicate experiments with 3–5 mice at each time point. $^{**}p < 0.01$; $^{***}p < 0.005$ for Foxp3$^-$ and Foxp3$^+$ cells from infected compared to naïve (d0) mice at each time point. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.005$ for Foxp3$^-$ compared to Foxp3$^+$ cells.

spleens of naïve and infected B6 mice on day 8 p.i. and migration to CXCL9 and CXCL10 was assessed using Transwell plates as previously described (19). Medium and the CCR7 ligand CCL21, highly expressed on naïve T cells, were used as negative and positive controls, respectively. As expected, CD4$^+$ T cells from naïve B6 mice migrated to CCL21 with a chemotaxis index 5-fold higher than cells from infected mice (**Supplementary Figure 2** and **Figure 4B**). Splenic CD4$^+$ T cells from infected B6 mice migrated in response to CXCL9 approximately three times more than cells from naïve mice with a significantly higher chemotaxis index, while no significant difference was observed for migration to CXCL10. To investigate if the difference in CXCR3 expression between effector CD4$^+$Foxp3$^-$ T cells and CD4$^+$Foxp3$^+$ Tregs during *P. chabaudi* AS infection described above correlated with differences in migration *in vitro* to CXCR3 ligands, cells were collected from the bottom chamber of the Transwell plate and surface stained for CD4 followed by intracellular staining for Foxp3 and the expression of Foxp3 was determined by flow cytometry. Approximately 13–17% of CD4$^+$ T cells from naïve mice that migrated to CXCL9 and CXCL10 were Foxp3$^+$ cells while <5% that migrated to CXCL21 were CD4$^+$Foxp3$^+$ (**Figure 4C**). Surprisingly, there were significant decreases in the frequency of CD4$^+$Foxp3$^+$ T cells that migrated to CXCL9 and CXCL10 on day 8 p.i. This was despite significant increases in the number of Tregs that expressed CXCR3 and in the level of CXCR3 expression as described above (**Figure 4C**). In contrast, more than 80% of CD4$^+$Foxp3$^-$ T cells from infected mice migrated to the C-X-C chemokines (data not shown). These findings indicate that expression of CXCR3 on effector CD4$^+$Foxp3$^-$ T cells may be important in trafficking of these cells to the spleen during malaria. In contrast, the ability of CD4$^+$Foxp3$^+$ Tregs to migrate to CXCR3 chemokines decreased significantly with infection. This observation is consistent with our observation of a higher number of CD4$^+$ Foxp3$^-$ T cells compared to CD4$^+$ Foxp3$^+$ Tregs in the infected spleen of B6 mice on day 8 p.i. (6).

The Course and Outcome of *P. chabaudi* AS Infection in WT vs. CXCR3$^{-/-}$ Mice

To determine if a deficiency in the chemokine receptor CXCR3 alters the course and outcome of blood-stage malaria, WT B6 and CXCR3$^{-/-}$ mice were infected with *P. chabaudi* AS and the percentage of parasitized RBC in the peripheral blood and survival were determined. Parasitemia increased to similar levels through day 8 p.i. in infected WT and CXCR3$^{-/-}$ mice (**Figure 5A**). Parasitemia peaked on day 10 p.i. in both genotypes, but the percentage of pRBC was significantly higher in infected CXCR3$^{-/-}$ compared to WT mice. Parasitemia was still significantly higher in CXCR3$^{-/-}$ than WT mice on day 11 p.i., but decreased to levels similar to WT mice by day 12 p.i. WT as well as CXCR3$^{-/-}$ mice cleared the infection by 3 weeks p.i. On day 10 p.i. when parasitemia peaked at high levels, 10% (1/10) of infected CXCR3$^{-/-}$ mice succumbed to infection

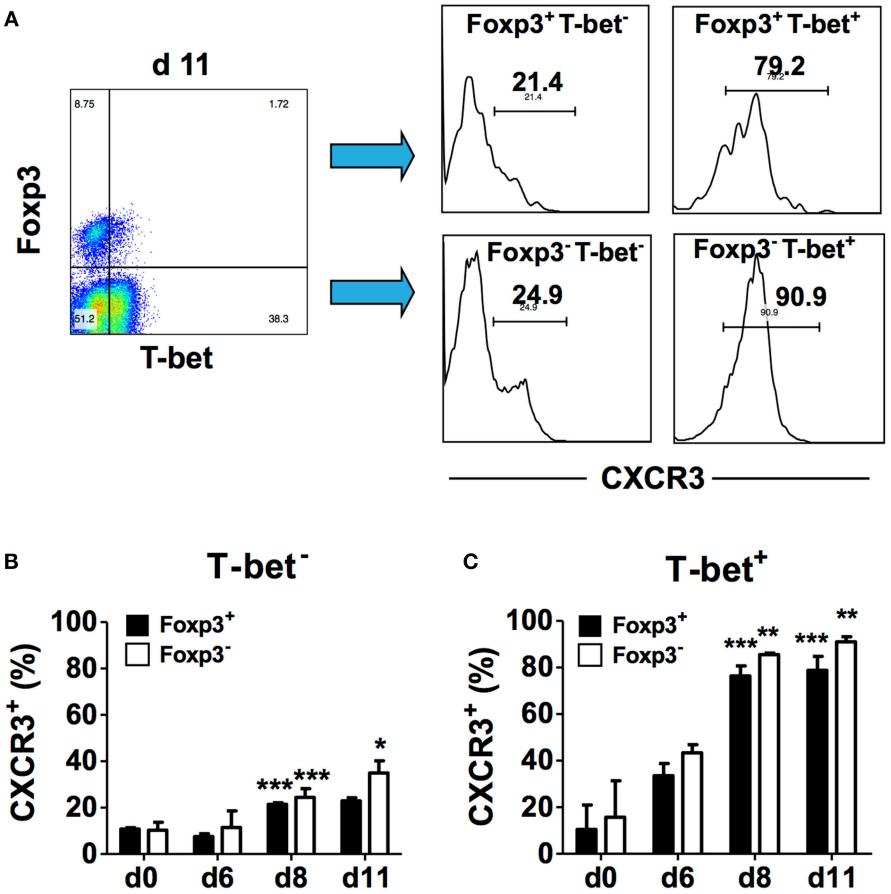

FIGURE 3 | T-bet expression is up-regulated on $CD4^+CXCR3^+$ T cells regardless of Foxp3 expression. Spleen cells were collected from naïve (d0) and infected B6 mice on the indicated days after i.p. infection with 1×10^6 *P. chabaudi* AS pRBC. The cells were surface stained with FITC-conjugated anti-$CD4^+$ and PE-conjugated anti-CXCR3 mAbs followed by intracellular staining with fluorochrome-conjugated anti-T-bet and anti-Foxp3 mAbs. The cells were gated on $CD4^+$ cells and analyzed for expression of T-bet, Foxp3, and CXCR3. **(A)** Representative plots from one infected mouse showing the gating strategy used to analyze CXCR3 expression within the different $CD4^+$ populations. **(B)** The frequency of $CXCR3^+$ cells within the $Foxp3^+$T-bet^- and $Foxp3^-$T-bet^- populations at various times p.i. **(C)** The frequency of $CXCR3^+$ cells within the $Foxp3^+$T-bet^+ and $Foxp3^-$T-bet^+ populations at various times p.i. Data are presented as mean ± SEM and are representative of one of two replicate experiments with 3–5 mice at each time point. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$ for CXCR3 expression on $Foxp3^-$ compared to $Foxp3^+$ cells.

(**Figure 5B**). Survival of CXCR3-deficient mice however was not statistically different from the survival of WT mice. WT as well as $CXCR3^{-/-}$ mice were immune to challenge infection (data not shown). Together, these data indicate that the ability to efficiently control parasite replication during acute *P. chabaudi* AS infection was decreased in the absence of CXCR3 but there was little or no effect on survival.

DISCUSSION

Here, we investigated the contribution of the chemokine receptor CXCR3 to immunity to blood-stage malaria infection. First, we identified the phenotype of $CD4^+$ T cells in the spleen that expressed this marker. We observed that the frequency and number of $CD4^+CXCR3^+$ T cells increased dramatically in the spleen of B6 mice during acute *P. chabaudi* AS infection. The timing of the increase coincided with increased $CD4^+$ T-bet^+IFN-γ^+ T cells in the spleen observed by our laboratory and other investigators (6, 8). We further observed that CXCR3 was up-regulated on effector $CD4^+Foxp3^-$ T cells as well as $Foxp3^+$ Tregs, implying these Tregs may be akin to the Th1-like Tregs described by Koch et al (19).

Previously, we observed there are significant increases in the frequency and number of $CD4^+Foxp3^+$ Tregs during acute *P. chabaudi* AS infection in B6 mice followed by a transient decrease until the infection is resolved (6). In the present study, we observed that $Foxp3^+$ Tregs expressing T-bet increased significantly. But the increases in both the frequencies and numbers were significantly lower than the responses of effector $CD4^+Foxp3^-$ T cells. Similar to effector $CD4^+$ T cells, a high frequency of $Foxp3^+$T-bet^+ Tregs also expressed CXCR3, further supporting the notion that these Tregs are likely Th1-like Tregs.

The findings described in the present manuscript further demonstrate that $CD4^+$ T cells from infected B6 mice migrated *in vitro* to the CXCR3 ligand CXCL9 with a significantly greater

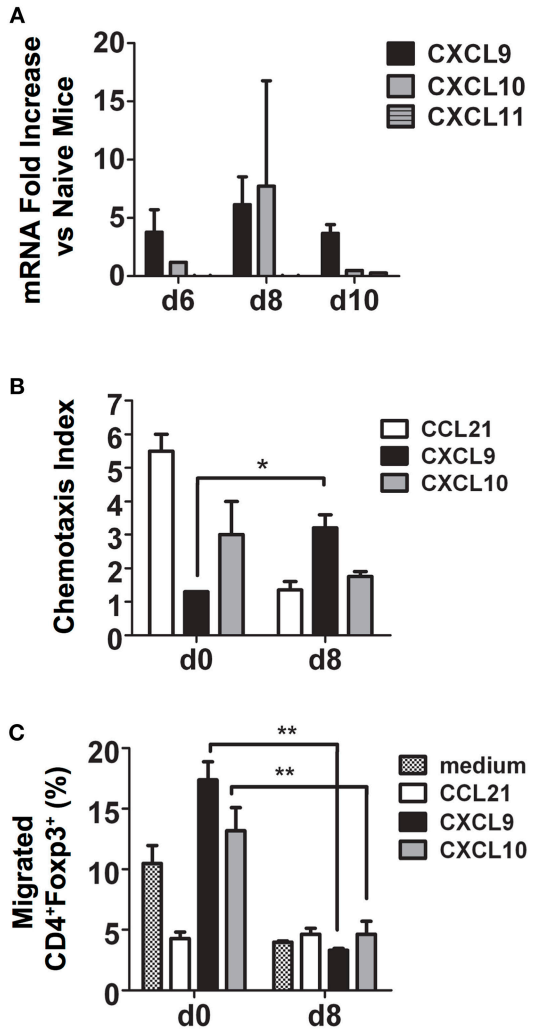

FIGURE 4 | During malaria, $CD4^+CXCR3^+$ T cells migrate to CXCL9 and CXCL10 and are predominately effector $CD4^+Foxp3^-$ T cells. Spleen cells were collected from naïve (d0) and infected B6 mice on the indicated days after i.p. infection with 1×10^6 *P. chabaudi* AS pRBC. **(A)** Total mRNA was extracted and chemokine expression was determined using the RT^2 Profiler PCR Array for mouse chemokines and receptors. The fold increase in mRNA expression of CXCL9, CXCL10, and CXCL11 in infected vs. naïve mice on days 6, 8, and 10 p.i. is shown. Data are presented as mean ± SEM and are representative of one of two replicate experiments with 3 mice at each time point. **(B)** $CD4^+$ T cells were purified from the spleens of naïve (d0) and infected B6 mice on the indicated days p.i., 5×10^5 cells were added to the top chamber of Transwell plates, and their chemotactic response to CCL21, CXCL9, and CXCL10 was determined. Migrated cells in the bottom chamber were collected, counted, and the chemotaxis index was determined as described in the Materials and Methods. Data are presented as mean ± SEM and are representative of one of two replicate experiments with five mice at each time point. $^*p < 0.05$. **(C).** Migrated cells from the bottom chambers of the Transwell plates described above were collected and surface stained with FITC-conjugated anti-CD4 mAb followed by intracellular staining for Foxp3. The cells were analyzed by flow cytometry to determine Foxp3 expression. The percentages of $CD4^+Foxp3^+$ Tregs that migrated to CCL21, CXCL9, and CXCL10 are shown. Data are presented as mean ± SEM and are representative of one of two replicate experiments with 5 mice at each time point. $^{**}p < 0.01$.

chemotaxis index than cells from naïve mice. $CD4^+Foxp3^+$ T cells also migrated to CXCR3 ligands, although to a lesser extent than effector $CD4^+$ T cells. Interestingly, a significantly greater percentage of $Foxp3^+$ Tregs from naïve B6 mice migrated to CXCR3 ligands than cells from infected mice, suggesting the possibility that expression of CXCR3 on Tregs is down-regulated during infection. However, additional experiments are required to address this possibility.

To address the *in vivo* role of CXCR3 during blood-stage malaria, we compared the course and outcome of *P. chabaudi* AS infection in WT (B6) and CXCR3-deficient mice. $CXCR3^{-/-}$ mice had significantly higher parasitemia levels around the time of peak parasitemia than WT mice. There were no significant differences in parasitemia levels during chronic infection and survival was similar in WT and $CXCR3^{-/-}$ mice. It should be noted that the increases in $CD4^+CXCR3^+$ T cells and $CD4^+IFN\text{-}\gamma^+$ T cells observed in the spleen of infected WT mice were coincident with high parasitemia levels in infected $CXCR3^{-/-}$ mice. This suggests a possible role for CXCR3 in controlling parasite replication during acute *P. chabaudi* AS infection. Additional studies however will be required to address the underlying defect in immune responses to blood-stage malaria in $CXCR3^{-/-}$ mice.

Despite studies demonstrating various Th cell subsets are activated during malaria, Th1 and Tfh cells have emerged as essential partners for the development of protective cell-mediated, antibody-dependent immunity to malaria with IFN-γ secreting Th1 cells playing a critical role (2). In addition to $CD4^+$ Th1 and Tfh cells, $Foxp3^+$ Tregs play an important regulatory role during malaria as a counterbalance to control excessive pro-inflammatory responses and limit immunopathology (4, 6, 7). The proliferation and suppressive activity of Tregs must be restrained during malaria as well as infection with other intracellular parasites to prevent uncontrolled replication of the pathogens (19). *P. chabaudi* AS-resistant B6 mice, used in this study, have a high ratio of effector $CD4^+$ T cells to $Foxp3^+$ Tregs during acute infection (6, 25). Availability of IL-2, important for the proliferation and survival of $Foxp3^+$ T Regs, is crucial for controlling Treg expansion during malaria as shown by increased $Foxp3^+$ Tregs and uncontrolled parasite replication in mice infected with *P. chabaudi* AS or *P. yoelii* after treatment with an IL-2-anti-IL-2 mAb complex (6, 7, 26).

To mediate their suppressive effects on effector $CD4^+$ T cells, it is critical that $Foxp3^+$ Tregs migrate to target tissues where Th1 and Tfh cells have accumulated (27). The spleen is the major site of immune responses to blood-stage malaria and is important for the accumulation of innate immune cells including dendritic cells and NK cells as well as $CD4^+$ T cells and B cells involved in adaptive immunity (23). Previously, we observed that $CD4^+Foxp3^+$ Tregs are located almost exclusively in the T cells areas of the white pulp (6). $Foxp3^+$ Tregs are known to express various chemokine receptors which direct their migration to sites of Th cell-mediated inflammation (28). Although previous studies showed that CXCR3 expression is up-regulated on splenic $CD4^+$ T cells during *P. berghei* ANKA infection, the phenotype of these cells was not defined in detail (15). Furthermore, the

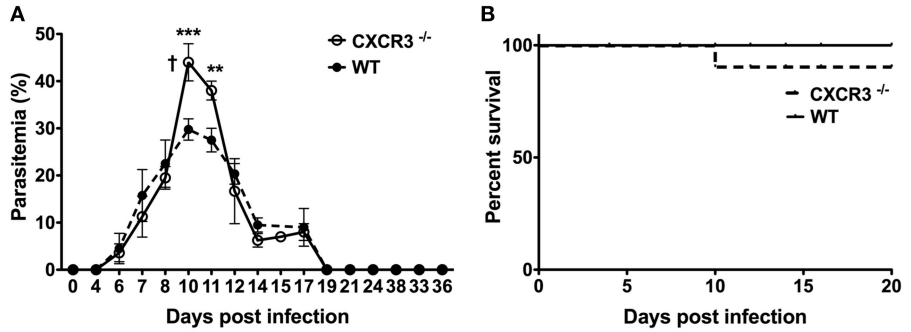

FIGURE 5 | The course and outcome of *P. chabaudi* AS infection in WT and CXCR3$^{-/-}$ mice. WT B6 and CXCR3$^{-/-}$ mice were infected i.p. with 1 × 10^6 *P. chabaudi* AS pRBC. **(A)** Course of parasitemia in WT and CXCR3$^{-/-}$ mice ($n = 5$ mice/group). Data are presented as mean ± SEM and are representative of one of two replicate experiments. †, indicates one mouse died on day 10 p.i. $^{**}p < 0.01$; $^{***}p < 0.005$. **(B)** Cumulative survival of WT and CXCR3$^{-/-}$ mice ($n = 10$ mice per group).

significance of the observation of increased CXCR3 expression on splenic CD4$^+$ T cells during malaria was unclear.

We observed that CXCR3 expression was associated with the ability of effector CD4$^+$ T cells to migrate *in vitro* to the IFN-γ-inducible C-X-C chemokines especially CXCL9. This finding is consistent with the importance of CXCR3 in Th1 cell recruitment to sites of inflammation and intracellular pathogen infection (19). We also observed that Foxp3$^+$ Tregs from naïve B6 mice and to a lesser extent from infected mice migrated *in vitro* to CXCL9 and CXCL10. This observation suggests the importance of CXCR3 expression in the trafficking of these cells to the spleen and possibly in regulating their suppressive function during malaria. A CXCR3-dependent mechanism has been shown to be important in Treg recruitment to sites of inflammation in the periphery and central nervous system as well as in an experimental model of liver disease (19, 29, 30). Together, these findings suggest that Foxp3$^+$ Tregs, like effector CD4$^+$ T cell subsets, respond to local cytokines by modifying their chemokine receptors including CXCR3 to promote their migration to sites of inflammation and infection where they function to suppress immune responses.

The transcription factor T-bet is the master regulator of Th1 cell generation and effector function and is required for IFN-γ secretion (18). T-bet is essential for increased CXCR3 expression by directly transactivating the *Cxcr3* promoter (21, 31). In the absence of T-bet, Th1 cells are unable to migrate *in vivo* to sites of inflammation and fail to migrate *in vitro* in response to the CXCR3 ligands CXCL10 or CXCL11 (21). Similar to conventional CD4$^+$ T cells, Foxp3$^+$ Treg populations have been identified with transcriptional profiles analogous to their effector Th cell counterparts and critical for their suppressive functions (28). Indeed, T-bet expression in Foxp3$^+$ Tregs has emerged as important for regulating Th1-mediated inflammation in various disease models in mice including in Foxp3-deficient Scurfy mice, airway hypersensitivity, autoimmune diabetes, and islet cell allograft rejection (19, 27, 32, 33). Moreover, disease modifying T-bet$^+$Foxp3$^+$ Tregs also express CXCR3 which facilitate their migration to sites of Type 1 inflammatory responses. Th1-like Foxp3$^+$ Tregs have been shown to produce IFN-γ and to express high levels of inhibitory molecules such as CTLA-4 as well as high IL-10 and TGFβ mRNA levels (19, 32, 34). Foxp3$^+$ Tregs expressing T-bet and CXCR3 have also been described in humans (35).

T-bet$^+$Foxp3$^+$ Tregs have been observed in mice infected with *Mycobacterium tuberculosis* and *Toxoplasma gondii* suggesting these cells may be important in infections with intracellular pathogens that induce highly polarized Th1 responses (19, 34, 36). In the present study, we observed that Foxp3$^+$ Tregs that co-express T-bet and CXCR3 increase significantly in the spleens of B6 mice during acute *P. chabaudi* AS infection. Recent studies indicate T-bet$^+$ Tregs increase during acute *P. vivax* infection while the frequency of Foxp3$^+$ Tregs that express T-bet is stable in patients after treatment (20). The exact role of T-bet$^+$Foxp3$^+$ Tregs in suppressing effector CD4$^+$ T cell responses during malaria has not yet been resolved.

In conclusion, we have identified a population of CD4$^+$Foxp3$^+$ Tregs that co-express CXCR3 and T-bet in the spleen during acute *P. chabaudi* AS infection. Altogether, our findings indicate that CXCR3 expression on effector CD4$^+$T-bet$^+$Foxp3$^-$ cells may contribute to the migration of these cells to the spleen during malaria suggesting a role for CXCR3 in immunity to *P. chabaudi* AS infection.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Mice were maintained and handled according to the guidelines of the Canadian Council on Animal Care and the McGill University Animal Care Committee (ACC). All procedures on experimental mice were approved by the McGill University ACC (Protocol #2015-3750).

AUTHOR CONTRIBUTIONS

FB, first author, designed and executed all the experiments, performed data analyses, prepared drafts of graphics for all relevant data, and was responsible for writing of first draft of the manuscript. CP, second author, co-supervised Dr. Berretta on the project, provided scientific input in the conception of some experiments, and contributed to the editing of the final manuscript. MS, senior author, is the Director of the laboratory and primary supervisor of Dr. Berretta. She provided scientific input in experimental design and to ensure that the data were soundly interrogated and interpreted. She was responsible for the final editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00425/full#supplementary-material

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REVIEW
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Development of B Cell Memory in Malaria

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A single exposure to many viral and bacterial pathogens typically induces life-long immunity, however, the development of the protective immunity to *Plasmodium* parasites is strikingly less efficient and achieves only partial protection, with adults residing in endemic areas often experiencing asymptomatic infections. Although naturally acquired immunity to malaria requires both cell-mediated and humoral immune responses, antibodies govern the control of malarial disease caused by the blood-stage form of the parasites. A large body of epidemiological evidence described that antibodies to *Plasmodium* antigens are inefficiently generated and rapidly lost without continued parasite exposure, suggesting that malaria is accompanied by defects in the development of immunological B cell memory. This topic has been of focus of recent studies of malaria infection in humans and mice. This review examines the main findings to date on the processes that modulate the acquisition of memory B cell responses to malaria, and highlights the importance of closing outstanding gaps of knowledge in the field for the rational design of next generation therapeutics against malaria.

Keywords: malaria, immunity, antibodies, memory B cells, inflammation

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B CELL IMMUNOLOGICAL MEMORY

Immunological memory refers to the ability of the vertebrate immune system to remember previously encountered antigens or pathogens and evoke an enhanced immune response to control infection. The capacity of the host to generate T and B cell memory underlies the basis of protective immunity induced by vaccination or after exposure to specific pathogens. The generation of T cell-dependent humoral immune memory in secondary lymphoid organs (**Figure 1**) typically begins following B cell engagement with its cognate antigen, which triggers their migration to the B cell follicle border to receive T cell help (1). Activated B cells then differentiate along one of three possible routes, leading to the rapid production of short-lived plasmablasts, generating germinal center (GC)-independent memory B cells (MBCs), or formation of GCs in B cell follicles (2, 3). GCs establish within a few days of initial antigen encounter and mature into two distinct micro-anatomical compartments: the dark zone, where B cell clones undergo proliferative expansion and somatic hypermutation of their immunoglobulin (Ig) genes, and the light zone, where B cells expressing high-affinity antibodies are selected and undergo class switch recombination (4–6). The GC reaction leads to the generation of affinity-matured MBCs and long-lived plasma cells that contribute to host protection against re-infection. Plasma cells migrate to the bone marrow and provide a continuous source of circulating high-affinity antibody (7), while MBCs recirculate in the blood and secondary lymphoid tissue (8) to induce a rapid effector response upon antigen re-encounter (9, 10).

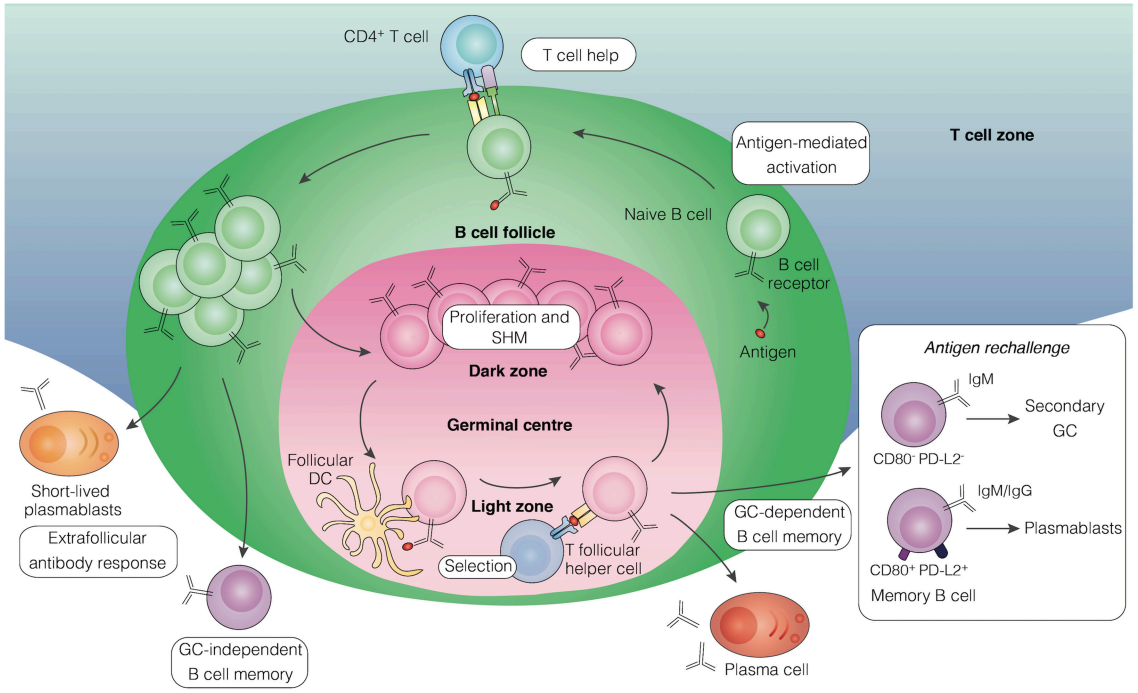

FIGURE 1 | Pathways leading to the development of B cell memory. Upon encounter with antigen, activated B cells in secondary lymphoid tissue receive helper signals from cognate $CD4^+$ T cells at the border of the B cell follicle and T cell zones. Some of the proliferating B cells differentiate into short-lived plasmablasts that initiate an extrafollicular antibody response, some develop into early memory B cells independently of GC formation, while others aggregate into the follicle to establish a GC. Within the GC, B cells undergo proliferation and somatic hypermutation in the dark zone, followed by affinity-based selection in the light zone with the help of T follicular helper cells and follicular dendritic cells. Long-lived plasma cells and memory B cells emerge from the GC reaction. Upon antigen rechallenge, memory B cells lacking expression of the surface molecules CD80 and PD-L2, mainly of the IgM isotype, can seed secondary GCs, whereas those expressing both molecules, comprising of IgM and IgG isotypes, predominantly generate plasmablasts. GC, germinal center; DC, dendritic cell; SHM, somatic hypermutation.

Comprehensive studies of MBC biology have led to the appreciation of substantial heterogeneity among the MBC compartment, consisting of distinct subpopulations with different effector capacity upon secondary challenge (11). In humans, the expression of unique memory-specific surface markers has been extensively used to identify and characterize MBCs. Surface expression of CD27 defines a subset of antigen-experienced MBCs in humans that are class-switched and bear Ig variable region mutations (12, 13). However, CD27 expression does not universally define all MBCs, as subsequent work identified an $CD27^-$ $CD21^-$ MBC population (14). These cells, coined as atypical MBCs in malaria, express several Fc receptor-like (FcRL) inhibitory receptors, including FcRL3 and 5 (15–17).

The development of novel labeling techniques to track antigen-specific B cells in murine models has enabled the functional characterization of MBCs expressing different antibody isotypes. Whereas, IgG MBCs have been found to preferentially differentiate into plasmablasts upon antigen rechallenge, IgM MBCs appear to have the capacity to re-enter secondary GC reactions but were not enhanced in plasmablast generation (18, 19). Recent studies have uncovered additional heterogeneity within the IgM and IgG MBC pools, proposing that MBCs lacking the memory markers CD80 and PD-L2, which primarily express IgM, are dedicated to reseeding GCs, while those expressing both markers, comprising of IgM and IgG isotypes, contribute mainly to immediate antibody-secreting function (**Figure 1**) (20). Consistently, IgM-expressing MBCs have recently been shown to exhibit considerable plasticity in their differentiation capacity following rechallenge (21). This division of labor in the MBC response has been proposed to support a rapid and effective response upon antigen rechallenge while concurrently permit the generation of new MBCs (11). How these findings in murine models relate to human settings is unclear, as there is still little information about the homology between mouse and humans MBC subsets with regards to their surface marker expression usage.

A ROLE FOR MEMORY B CELS IN NATURALLY ACQUIRED IMMUNITY TO MALARIA

The global burden of malaria has more than halved since the turn of the century due to renewed eradication efforts, but progress has recently stalled as current intervention strategies are confronted with several major challenges, including the emergence of anti-malarial drug and insecticide resistance

(22). Of the six *Plasmodium* species known to infect humans, *P. falciparum* continues to account for the majority of deaths, whereas recurrent *P. vivax* episodes are a significant source of morbidities. Disease syndromes of malaria range from fever to more severe complications including acute pulmonary oedema, jaundice, severe anemia, hypoglycaemia, acidosis, and cerebral malaria (23). The pathogenesis of malarial disease is thought to arise from the concerted effects of host and parasite mechanisms, including the sequestration of blood-stage parasites in microvasculature, and local and systemic inflammation induced by the parasites and their toxic products (24, 25).

Early epidemiological observations by Robert Koch in malaria-endemic populations described that natural immunity to malaria can be achieved, but requires years of repeated exposure to *Plasmodium* parasites (26). Children living in high transmission regions become immune to the most severe forms of malaria after relatively few symptomatic infections (27–29), but remain at risk of uncomplicated malaria. After years of repeated infections with age, protection from successive malaria episodes or "clinical immunity," is acquired by the ability to substantially reduce parasite burdens (30–35). This form of protection is not paralleled by sterile immunity that prevents re-infection (36), and adults continue to be experience low-density, asymptomatic infections throughout life (37). Naturally acquired clinical immunity to malaria targets blood-stage parasites and requires antibodies, as demonstrated by studies in which the transfer of purified IgG from malaria-immune adults to children with symptomatic malaria rapidly reduced parasitemia and fever (38). Together, these observations have led to the hypothesis that the slow and imperfect acquisition of immunity to malaria reflects in the development of MBCs, and this topic has been the subject of several studies including mouse infection models as well as human settings. Here we review our current understanding on the salient features of the development of humoral immunity to malaria infection, and highlight some of the outstanding questions regarding the cellular mechanisms that underlie the slow acquisition of clinical immunity.

ANTIBODY RESPONSES TO BLOOD-STAGE MALARIA

The paramount importance of antibodies in controlling blood-stage malaria infection was proven by seminal passive-transfer experiments, in which IgG from *P. falciparum* clinically immune adults protected non-immune children from high parasitemia and clinical symptoms (38, 39). Numerous immuno-epidemiological studies subsequently demonstrated that high antibody levels against specific blood-stage parasite antigens correlate with protection from disease (40–46). Antibodies may control the development of clinical symptoms by targeting the invasion and growth of the merozoite form of the blood-stage parasite and redirect their clearance by phagocytic cells via Fc and complement receptors (47). Additionally, antibodies directed against parasite antigens expressed on infected erythrocytes can promote opsonic phagocytosis, block microvasculature adherence, disrupt rosette formation with uninfected cells, and prevent erythrocyte rupture and parasite egress (47).

Antibodies may target a number of highly polymorphic and functionally redundant antigens expressed by *Plasmodium* parasites (48), which may represent a potential mechanism by which the parasite effectively evades the human immune system via antigenic variation (49). Asymptomatically-infected individuals who fail to mount an antibody response against *P. falciparum* has been shown to predict increased susceptibility to clinical disease (50, 51). In parallel, individuals detected with multi-clonal *P. falciparum* infections in the dry season have been associated with subsequent protection from febrile malaria (52), suggesting that the presence of persisting parasites enhance antibody recognition and enable cross-reactive responses. This supports the notion that clinical immunity may depend on the cumulative acquisition of a repertoire of antibodies to a diverse range of parasite antigens or development of cross-species antibody responses (53–55). Indeed, the breadth of parasite-specific antibody responses have been identified to increase with age in endemic populations (56–58), and the antibody repertoire diversifies rapidly during infancy but plateaus in toddlers (59). Moreover, antibodies with broad reactivity against *P. falciparum* that carry a gene insertion derived from the collagen-binding protein LAIR1, have been shown to undergo somatic hypermutation that increase binding to infected erythrocytes (60). LAIR1 insertions have been further revealed to represent a relatively common mechanism of antibody diversification in African individuals, and that broadly-neutralizing antibodies against *Plasmodium* arise from these low-affinity precursors over time (61). While antigenic variation has been proposed to explain the slow acquisition immunity to malaria, there is also an increasing body of evidence suggesting that antibody responses to malaria are poorly generated. In malaria-endemic areas, substantial declines have been reported in *Plasmodium*-specific antibody responses to low or undetectable levels within months, and even weeks of a clinical episode after reduced parasite exposure, despite an initial robust response (56, 57, 62–69). Studies modeling the longevity of *P. falciparum* merozoite-specific IgG antibodies have estimated average half-lives of <10 days in children recovering from clinical malaria (67). Similarly, short antibody half-lives ranging from only 2–7 weeks has been reported in asymptomatic children during the dry season in The Gambia (68), which contrasts dramatically with those of antibody responses to viral and bacterial antigens such as vaccinia, measles and tetanus that reportedly persist for decades following a single exposure (70–72). It is possible that antibody responses measured following a clinical malaria episode may reflect the output of short-lived antibody-secreting cells, which typically produce an immediate but transient wave of antibodies to control infection. In line with this idea, antibody-secreting cells have been detected transiently in Ugandan children immediately following acute malaria, and were found to proportionally increase again upon a second clinical episode (73). Modeling studies extended these observations, estimating the longevity of both short- and long-lived antibody-secreting cells in African children to range from 2–10 days, and 3–9 years, respectively (74). Thus, together these findings suggest that a

long-lived humoral response to malaria infection can potentially be sustained after decay of transient antibody-secreting cells. More detailed mechanistic investigations are much needed to determine how parasite-specific antibody responses are sustained over time and the factors that modulate the generation and maintenance of antibody-secreting cells to infection.

THE ACQUISITION OF MEMORY B CELLS TO NATURAL MALARIA INFECTION

Several studies have now shown the induction of *Plasmodium*-specific MBCs in response to malaria infection; although individuals exposed to high seasonal transmission have been reported to induce only low frequencies of MBCs or to lack detectable MBCs, even after exposure to parasitic loads sufficient and capable of inducing antibody responses (57, 75, 76). Consistent with the slow acquisition of antibody responses in endemic settings, the prevalence and breadth of *Plasmodium*-specific MBCs appear to develop incrementally with age and exposure (57, 59, 77). Longitudinal studies of children and young adults in an area of high seasonal transmission in Mali demonstrated a delayed, age-associated development of MBCs specific for *P. falciparum* merozoite antigens despite repeated infections annually (57). Moreover, the prevalence of MBCs acquired by children following acute malaria appeared to diminish substantially within the 6-month dry season, contrasting with the stable frequency of MBCs to tetanus toxoid vaccination in the same subjects (57). While seasonal transmission prevented the longevity of these cells to be determined beyond the dry season, this finding implies an impaired maintenance or generation of MBCs in children exposed to high transmission as they acquire clinical protection.

In contrast, individuals residing in areas of low transmission or subjected to fewer clinical episodes have been shown to generate stable *Plasmodium*-specific MBCs without frequent boosting (77–85). The frequencies of *P. falciparum*-specific MBCs detected in Thai adults that had experienced limited episodes reportedly remained stable over time, with an estimated half-life of approximately 7.5 years (81). Similarly, malaria-specific MBCs have been described to be well-maintained in individuals with a history of acute malaria but have since lived in the absence of persistent infection (83, 84). In parallel, low levels of exposure can effectively sustain parasite-specific antibody responses (79, 82, 84, 86, 87), with antibody half-lives estimated to be substantially longer than that of highly-exposed individuals, ranging from months to years (32, 81, 88), suggesting sustained antibody production from long-lived plasma cells. Collectively, these findings reveal that MBCs can be generated and sustained following a limited number of clinical episodes, while repeated infections in endemic areas could have a detrimental effect on the generation of B cell memory.

The characterization of malaria-specific MBCs to date has relied predominantly on *in vitro* stimulation and differentiation of circulating MBCs into antibody-secreting cells followed by detection of antigen-specific clones by ELISPOT assays. This approach precludes phenotypic analysis of the malaria-specific MBC compartment. More detailed investigations are needed to determine the ontogeny of detected MBCs and whether they contribute to effective clinical immunity in malaria-exposed individuals. Whereas, the induction of IgG-expressing MBCs has been the primary focus over the past several years, a few studies have identified IgM MBCs in malaria-exposed individuals and in malaria mouse infection models (59, 89, 90), with those induced by murine malaria found to rapidly differentiate into plasmablasts upon antigenic restimulation (89). Interestingly, MBCs isolated from malaria-exposed individuals have been described to have undergone Ig somatic hypermutations (76), and accumulate further mutations upon acute malaria, with IgM being the dominant isotype expressed prior to re-infection (59).

The use of murine infection models has also provided insight into the development of B cell memory to malaria. Although murine infection does not mirror all the features of human malaria, there are genetic and phenotypic parallels between the human parasite and rodent counterparts (91, 92). A few studies have detected the development of IgG memory B cells following non-lethal *P. chabaudi* infection (89, 90, 93, 94), associated with efficient generation of secondary GCs and enhanced control of re-infection (90, 95). In contrast, *P. yoelii* blood-stage infection has been suggested to ablate vaccination-induced MBCs (96), and further reduces the development of mature, isotype-switched MBCs against pre-erythrocytic parasite antigen, which was associated with the induction of pro-inflammatory cytokines and chemokines that may hinder effective T and B cell interactions (97).

IMMUNOLOGICAL PROCESSES MODULATING THE INDUCTION OF MEMORY B CELLS IN MALARIA

T follicular helper (Tfh) cells are a crucial subset of $CD4^+$ T cells that orchestrate B cell memory development by providing crucial survival and differentiation signals to B cells during the GC response (98), and have been shown to be important for the control of *Plasmodium* infection (99–105). The development of Tfh cells has not been extensively investigated in human malaria infection, however, a recent study identified the induction of T helper 1 (Th1) cell-like $CXCR3^+PD\text{-}1^+$ Tfh cells in the circulation of Malian children following acute *P. falciparum* malaria (106). Circulating Tfh cells have been proposed to be a surrogate measure of GC-derived Tfh cell responses to human infection (107, 108) and thus constitutes a valuable proxy to investigate Tfh cell biology in human settings. Circulating Tfh cells in malaria-exposed children were shown to express the Th1 lineage-defining transcription factor T-bet, and had limited functional capacity to support MBC responses *in vitro* (106). $CXCR3^+PD\text{-}1^+$ Tfh cells have also been identified in Brazilian adults during acute *P. vivax* malaria (109). Interestingly, their proportions positively correlated with repeated malaria episodes (109), implying that circulating Tfh cells accumulate with sustained parasite exposure. However, whether or not the detected Tfh cells facilitate the induction of MBCs that confer

protection to disease over time remains to be investigated in larger cohorts and different transmission settings. In addition, circulating follicular regulatory T (Tfr) cells have been postulated to interfere with Tfh cell responses to infection (110). Tfr cells expressing the inhibitory molecule CTLA-4, have been detected at increased frequencies after acute malaria in Malian children (110). Future studies will be important in determining if the transition between susceptibility to clinical disease and asymptomatic infection is associated with the development of functional Tfh cell responses that support the induction of B cell memory populations.

Similar to human malaria, $CD4^+$ T cells induced by murine infection have been described to express a dual Th1- and Tfh-associated phenotype, including chemokine receptor CXCR5, and cytokines IL-21 and IFN-γ (99, 111, 112). Precursor Tfh cells induced in response to *P. berghei* severe malaria infection also display a Th1 cell-like phenotype, characterized by the upregulation the chemokine receptor CXCR3 and transcription factor T-bet (113). Moreover, the expression of T-bet impaired the differentiation of Tfh cell precursors into mature Tfh cells, which significantly suppressed the development of GC B cells, plasma cells and MBCs (113). Notably, genetic ablation of T-bet or neutralization of the pro-inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor (TNF) promoted Tfh cell differentiation, and restored previously impaired GC responses, demonstrating that inflammatory responses associated with the induction of symptomatic malaria reduce the magnitude of MBC responses by modulating effective Tfh cell development (114). In support of this concept, exogenous IFN-γ has been shown to reduce Tfh and GC B cell responses to *Plasmodium yoelii* (102), and the blockade of CXCR3-mediated signals was found to enhance precursor Tfh cell accumulation in the spleen of malaria-infected mice, thereby favoring parasitic clearance (115). Other inflammatory pathways such as type I IFN signals have also been recognized to indirectly limit Tfh and GC B cell responses to infection (116, 117), although the precise mechanisms by which this occurs is not yet clear. Recent studies suggest that the damage-signal sensor P2X7 (118), IFN regulatory factor 3 (119), and changes in metabolism and proliferation, as well as altered gene expression of chemokine receptors (120) influence the bifurcation of Tfh and Th1 cell differentiation during murine infection. Whereas, inflammatory responses to malaria appear to dampen the magnitude of the MBC response to infection (114), effective control of blood-stage malaria and its associated pro-inflammatory responses appears to permits normal progression of Tfh cell development (113, 121), giving rise to T cell memory that responds to secondary infection (112).

Whereas, it is reasonable to assume that the aforementioned modulation of Tfh cell function by inflammatory pathways elicited in response to infection is responsible for the reduced B cell responses observed during acute infection, a direct effect of IFN-γ-mediated signaling or intrinsic expression of T-bet in B cells cannot be ruled out. *P. yoelii* infection has recently been shown to induce a subset of B cells to express the transcription factor T-bet, and deletion of B cell-specific IFN-γ receptor or T-bet deletion elevated antibody responses to infection (122, 123). Further work to establish if inflammation directly modulates the differentiation and functional capacity of B cells is needed to address this question.

ATYPICAL MEMORY B CELLS: FRIENDS OR FOES?

There is evidence that *Plasmodium* parasites can directly modulate B cell function. *In vitro* studies have shown that the parasite can directly activate human naive B cells via a cysteine rich inter-domain region of *P. falciparum* erythrocyte membrane protein 1 (124, 125) and downstream toll-like receptor signaling (126), which may lead to non-specific polyclonal activation of B cell responses. CD27-expressing B cells were observed to be the major responding population (124, 125), suggesting a potential impact on the MBC compartment. In line with this idea, acute *P. falciparum* infection has been reported to modulate systemic mediators of B cell activation and survival (127, 128), which has been associated with an early proliferation of MBC subsets, prior to the induction of GC responses following experimental human infection (128), supporting a potential role of bystander activation of non-specific MBC subsets that enables parasite evasion.

Insight into the notion that B cell memory induction in malaria may be dysregulated surfaced upon the characterization of an expansion of MBCs exhibiting an atypical phenotype following persistent malaria exposure (17, 84, 129, 130). Originally described as an exhausted subset of MBCs implicated in humoral deficiencies in HIV infection (131), a phenotypically similar $CD27^-CD21^-$ circulating MBC subset notable for their expression of inhibitory receptors, was detected in *Plasmodium*-infected individuals following acute malaria in Brazil (109), Gabon (132), Ghana (85), Gambia (77), India (133), Kenya (84, 130), Mali (15, 17, 129, 134, 135), Papua New Guinea (136, 137), Thailand (78), and Uganda (16, 73, 138), and further exacerbated by HIV co-infection (139, 140). Studies to date corroborate that the accumulation of this subset is influenced by the level of parasite exposure. Higher frequencies of atypical MBCs were found in adults compared to children with shorter exposure histories (17). Similarly, Malian adults from high transmission settings were found to have higher frequencies of these cells than Peruvian individuals residing in a low transmission area (129). A decline in the atypical MBC pool was observed during 12 months without malaria transmission (84), further substantiating a role of persistent parasite exposure in the maintenance of this population.

Recent work has investigated the function of these atypical MBCs and their relationship to classical MBCs. Evaluation of the variable gene repertoires of atypical MBCs proposed that they share a common developmental precursor to classical MBCs (15, 132, 141), however, emerging work support that atypical MBCs are phenotypically and functionally different to classical MBCs. There is still conflicting evidence on the effector capacity of atypical MBCs from malaria-infected individuals. Whereas, some support the notion that atypical MBCs are

capable of secreting neutralizing antibodies (132), subsequent work contend their lack of active antibody secretion (15, 16). Atypical MBCs have been reported to express lower levels of surface IgG (136), possess reduced B cell receptor signaling, as well as impaired interleukin (IL)-6, IL-8 and antibody production *in vitro* (15, 16). Atypical MBC frequencies in malaria-exposed individuals have been shown to be positively correlated with pro-inflammatory cytokine levels such as IL-8 (136), and their expression of the inhibitory molecule FcRL5 has been proposed to be a marker of dysfunction associated with increased malaria exposure (16). FcRL5-expressing atypical-like MBCs in healthy individuals have been distinguished by their higher expression of inhibitory receptors such as PD-1 and transcription factors *Tbx21, Bcl-6*, and *Sox5* and blunted proliferation capacity compared to FcRL5$^-$ B cells (142). In line with these findings, recent studies revealed that atypical MBCs in Malian children express *Tbx21* (134), which encodes the Th1 transcription factor T-bet (143). Emerging evidence that T-bet becomes up-regulated in various disease contexts in subsets of B cells that share many features in common with atypical MBCs found in malaria-infected individuals. T-bet-expressing B cells have been associated with both protective and pathogenic roles depending on specific settings, which include bacterial (21), parasitic (144–146) and viral infection (147–153), cancer (154–156), autoimmune conditions (157–164), and aging (165). In malaria, atypical MBCs expressing high levels of T-bet have also been found to express decreased levels of the co-stimulatory molecule CD40 and reduced phosphorylation of B cell receptor signaling molecules (134), suggesting that T-bet expression might reduce B cell effector function. In line with this concept, the frequency of malaria episodes was found to be associated with increased T-bet^{hi} MBC in a small group of children (134). On the other hand, longitudinal cohort studies have reported that low parasite density malaria is associated with persistence (78) or accumulation of atypical MBCs over time, which raises the possibility that atypical MBCs contribute to developing immunity (73, 138), although T-bet expression was not examined in these cells. Furthermore, similar atypical MBCs that express FcRL5 have been recently detected in *P. chabaudi*-infected mice (166, 167), but appear to display normal features of proliferation and Ig expression, representing a short-lived population of activated MBCs (167). Collectively, a causal relationship between atypical MBCs and immune protection or disease progression remains unclear, and more studies are urgently needed to determine the functional significance of atypical MBC expansion in the acquisition of humoral immunity to malaria and the precise contribution of the transcription factor T-bet in shaping atypical MBC function.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Emerging evidence both in human field studies and murine infection models are beginning to address how the induction of humoral immune responses is compromised during acute malaria, and the consequences of these processes for the establishment of long-term immunological B cell memory. There is now considerable evidence that exposure to *Plasmodium* parasites is associated with altered proportions of circulating memory B cells, including an expansion of atypical memory B cells. Further studies to unravel the complexity of this diverse memory B cell compartment, in terms of origin, function and protective capacity of different subpopulations are urgently needed. Information about the level of homology between human and mouse memory B cell subsets is lacking, particularly in relation to their surface marker expression patterns, which makes it difficult to infer how findings in malaria infection models translate into human settings. Gene expression analysis studies, including how transcription factors and the cytokine environment influence these processes might be required to overcome these issues and establish functional correlations between human studies and much needed mechanistic work in mice.

Given the critical importance of antibodies and long-lived humoral memory in immunity to malaria, an in-depth understanding of the factors that delay their development is undoubtedly required to inform the design of targeted therapeutic strategies to enhance immune responses to the parasite and protect against disease susceptibility. Detailed characterization of the immune processes by which B cell memory to malaria is generated and the specific effector populations required to confer protection, will undoubtedly benefit vaccine development and optimisation efforts, especially in light of the modest efficacy levels achieved to date with current vaccination regimes at a population level (168, 169).

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Low Levels of Human Antibodies to Gametocyte-Infected Erythrocytes Contrasts the PfEMP1-Dominant Response to Asexual Stages in *P. falciparum* Malaria

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Vaccines that target *Plasmodium falciparum* gametocytes have the potential to reduce malaria transmission and are thus attractive targets for malaria control. However, very little is known about human immune responses to gametocytes present in human hosts. We evaluated naturally-acquired antibodies to gametocyte-infected erythrocytes (gametocyte-IEs) of different developmental stages compared to other asexual parasite stages among naturally-exposed Kenyan residents. We found that acquired antibodies strongly recognized the surface of mature asexual-IEs, but there was limited reactivity to the surface of gametocyte-IEs of different stages. We used genetically-modified *P. falciparum* with suppressed expression of PfEMP1, the major surface antigen of asexual-stage IEs, to demonstrate that PfEMP1 is a dominant target of antibodies to asexual-IEs, in contrast to gametocyte-IEs. Antibody reactivity to gametocyte-IEs was similar to asexual-IEs lacking PfEMP1. Significant antibody reactivity to the surface of gametocytes was observed when outside of the host erythrocyte, including recognition of the major gametocyte antigen, Pfs230. This indicates that there is a deficiency of acquired antibodies to gametocyte-IEs despite the acquisition of antibodies to gametocyte antigens and asexual IEs. Our findings suggest that the acquisition of substantial immunity to the surface of gametocyte-IEs is limited, which may facilitate immune evasion to enable malaria transmission even in the face of substantial host immunity to malaria. Further studies are needed to understand the basis for the limited acquisition of antibodies to gametocytes and whether vaccine strategies can generate substantial immunity.

Keywords: gametocytes, PfEMP1, antibodies, malaria, *P. falciparum*, immunity

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INTRODUCTION

Strategies to develop highly effective vaccines against malaria remain a high priority. In particular, the development of vaccines that interrupt malaria transmission, known as transmission-blocking vaccines, is currently recognized as a key goal to sustain long-term malaria elimination (1).As such, there is a renewed interest in gametocytes, the sexual, transmissible stages of *Plasmodium falciparum*, which involves distinct parasite forms that establish infection in the mosquito vector. Currently, the advancement of transmission-blocking vaccines is hampered because very little is known about immune responses to sexual-stage antigens.

The sexual cycle of *P. falciparum* begins when immature asexual blood-stage parasites undergo commitment to produce gametocytes. The early gametocyte-IE stages (I–IV) are sequestered and develop within organs such as the spleen and bone marrow (2–4). Upon maturity to stage V, gametocyte-IEs are released into the peripheral circulation and taken up by feeding mosquitoes. During asexual development, *P. falciparum* remodels the host erythrocyte through the expression of knobs on the IE surface, which present the major surface antigen PfEMP1 (5). Specific interactions between PfEMP1 and host endothelial receptors enable the vascular sequestration of asexual parasites in various microvascular beds [reviewed in (6)]. However, knobs are absent from the surface of gametocyte-IEs (7), and PfEMP1 has not been detected on the surface of gametocyte-IEs, suggesting that gametocyte commitment is accompanied by the silencing of *var* genes (7). Other antigens have been identified on the surface of asexual IEs (including RIFIN, STEVOR, and SURFIN), with some evidence they are expressed by gametocyte-IEs (8).

Antibodies against circulating gametocytes have the potential to reduce malaria transmission efficiency by mediating parasite clearance within the human host or inhibiting further development of exflaggelated gametocytes within the mosquito midgut [reviewed in (9)]. However, knowledge of human antibodies against gametocyte-IEs is currently very limited [reviewed in (9)]. One study reported naturally-acquired antibodies recognized the surface of immature gametocyte-IEs (10). In contrast, other studies reported a lack of antibodies to immature stages, but some antibodies to mature stage V gametocyte-IEs (11–13). The target of these antibodies is unknown. In contrast, antibodies to gametocyte surface antigens, such as Pfs230 and Pfs48/45, are acquired relatively quickly and increase with cumulative exposure (14–18). As malaria transmission may still occur despite substantial acquired immunity to asexual parasites, it is likely that gametocyte-IEs do not share the surface antigens that elicit this immunity.

In order to address knowledge gaps in understanding human transmission-blocking immunity, we quantified antibodies to gametocyte-IEs compared to asexual IEs, and investigated the potential basis for differences in antibody reactivity to different developmental stages.

MATERIALS AND METHODS

A detailed description of methods is included in **Supplementary Materials**.

Study Population and Ethics Statement

Plasma were collected at two study sites in Kenya (Kanyawegi and Chulaimbo) from individuals aged 0.5–79 years, as described (19, 20).

Ethics approval was obtained from Alfred Hospital Human Research and Ethics Committee, Australia, Institutional Review Board for Human Investigation at University Hospitals of Cleveland for Case Western Reserve University, USA and the Ethical Review Committee at the Kenya Medical Research Institute. Written informed consent was obtained from all study participants or their parents or legal guardians.

P. falciparum Culture and Gametocyte Isolation

P. falciparum was maintained in continuous culture and synchronized as described (21). Isolates 3D7vpkd and 3D7-SBP1KO, with inhibited PfEMP1 surface expression, were generated as previously described (21, 22). Gametocytes were generated according to established protocols (11, 12), with the modification of using heparin (100 ng/mL) throughout gametocyte development to inhibit asexual replication (23).

Measuring Antibodies to the IE Surface

Measuring IgG binding to the IE surface was performed by flow cytometry as previously described (21). IgG levels are expressed as the geometric mean fluorescence intensity (MFI; arbitrary units).

Antibodies to Recombinant Proteins

We expressed a modified form of recombinant Pfs230D1H (24), a truncated form of Pfs230 containing the first 6-cys domain of Pfs230 (termed Pfs230D1M) expressed in the mammalian HEK293 cells. IgG binding to recombinant Pfs230D1M was measured using standard ELISA methods (25).

Immunofluorescence Microscopy

Imaging of thin blood smears of stage V 3D7 gametocyte-IEs was performed as previously described (21) and processed using Photoshop CS6 (Adobe).

Statistical Analyses

Non-parametric analytical methods were used to evaluate antibody results. Differences in antibody levels between trophozoite-IEs and gametocyte-IEs were assessed using a paired Wilcoxon signed rank test. Statistical analyses were performed using Prism version 7 (GraphPad Software Inc).

RESULTS

Naturally-Acquired Human Antibodies to Gametocyte-IEs Are Markedly Lower Than to Asexual Trophozoite-IEs

We conducted a time-course assay based on the different developmental stages of gametocytes to assessed naturally-acquired antibodies. Thin blood smears were prepared and visualized by Giemsa staining to confirm the gametocyte stages (**Figure 1A**). We measured the level of human antibodies to the surface of stages II-V gametocyte-IEs by an established flow cytometry-based assay (21) in malaria-exposed individuals residing in Kenya ($n = 21$; children $n = 11$ and adults $n = 10$), compared to antibody binding to mature pigmented asexual trophozoite-IEs. There were noticeably low levels of antibody reactivity to the surface of gametocyte-IEs across all developmental stages (**Figure 1B**, **Figure S1**). Further, there was no significant difference in antibody levels observed between children and adults across different developmental gametocyte-IE stages (**Figure S1F**). In contrast, using the same selection of plasma samples ($n = 21$; children $n = 11$ and adults $n = 10$), high antibody reactivity was measured against surface antigens of trophozoite-IEs (**Figure 1B**, **Figure S1**). We confirmed our findings by measuring antibodies to stage V gametocyte-IEs in a second cohort of malaria-exposed Kenyan adults ($n = 20$; **Figure S2**). Similarly, all individuals had markedly lower antibody levels to stage V gametocyte-IEs compared to trophozoite-IEs (**Figures S2A,B**). Furthermore, increasing the plasma concentration from both human cohorts (1:2 dilution) used in assays did not substantially increase reactivity (**Figures S2C,D**). Our findings show that antibodies induced during natural malaria exposure have little reactivity to the surface of gametocyte-IEs across all stages.

PfEMP1-Dominant Antibody Response in Asexual Trophozoite-IEs Contrasts the Low Response to Stage V Gametocyte-IEs

We hypothesized that the high reactivity to trophozoite-IEs, compared to gametocyte-IEs may be explained by antibodies targeting PfEMP1. Using genetically-modified *P. falciparum* with suppressed PfEMP1 expression, we found that PfEMP1 was a dominant target of naturally-acquired antibodies to the surface of asexual trophozoite-IEs (**Figures 1C,D**, **Figure S3A**) using the same selection of plasma samples from Kenyan children and adults ($n = 21$; children $n = 11$ and adults $n = 10$). This is consistent with our previous reports (21, 22). This was demonstrated by the greatly reduced reactivity of antibodies to 3D7vpkd and 3D7-SBP1KO IEs compared to 3D7 parental IEs. To better understand the magnitude of antibody responses to gametocyte-IEs, we compared antibodies to stage V gametocyte-IEs with asexual trophozoite-IEs that have reduced PfEMP1 expression (using 3D7vpkd and 3D7-SBP1KO). The level of antibodies to stage V gametocyte-IEs were substantially lower compared to trophozoite-IEs from 3D7 parental (71.1% lower; **Figures 1C,D**; $p < 0.0001$) and 3D7vpkd (55.9% lower; $p < 0.0001$). There was minimal difference in antibody levels to stage V gametocyte-IEs and 3D7-SBP1KO ($p = 0.02$). Prior studies suggested that there is still low levels of PfEMP1 expression on the surface of 3D7vpkd IEs (21, 22), which may explain the higher antibody reactivity to 3D7vpkd compared to 3D7-SBP1KO and gametocyte-IEs. These findings suggested that the lack of PfEMP1 on gametocyte-IEs is likely to be a major reason for the low reactivity of antibodies compared to trophozoite-IEs.

Gametocytes Without the IE Membrane Are Recognized by Acquired Human Antibodies

We quantified the level of naturally-acquired antibodies to surface antigens expressed on the gametocyte plasma membrane. The IE membrane was removed by saponin treatment, and thin blood smears with Giemsa staining was used to confirm that gametocytes remained intact post-saponin treatment (**Figure 2A**). The level of antibodies to 3D7 stage V gametocytes and gametocyte-IEs was measured in a subset of Kenyan individuals ($n = 5$ from samples tested in **Figure 1**; children $n = 3$ and adults $n = 2$). High levels of antibodies were observed to 3D7 gametocytes, but not 3D7 gametocyte-IEs (**Figures 2B**,**C**, **Figure S3B**; reactivity 92.3% higher; $p = 0.06$).

To further assess antibodies to gametocytes, we measured antibodies to recombinant Pfs230, a major gametocyte surface antigen and vaccine candidate (24). We measured IgG reactivity in the same selection of Kenyan individuals used in assays of trophozoite-IEs and gametocyte-IEs ($n = 21$ from **Figure 1**; children $n = 11$ and adults $n = 10$). The majority of individuals (80.9%) were positive for antibodies to Pfs230D1M (**Figure 2D**; **Figure S4**) and there was a positive correlation between IgG binding to Pfs230D1M and to whole gametocytes ($r_s = 0.9$; **Figure 2E**). Further, immunofluorescence microscopy demonstrated that anti-Pfs230 rabbit antibody labeled native Pfs230 expressed on the surface of stage V gametocytes (**Figure 2F**). Together, these findings suggest that individuals do acquire antibodies to sexual stage parasites, but there is very limited acquisition of antibodies target antigens on the gametocyte-IE surface.

DISCUSSION

There was limited acquisition of antibodies to antigens on the surface of gametocyte-IEs, despite study subjects having high levels of antibodies to asexual trophozoite-IEs, as well as substantial antibody reactivity to the major gametocyte antigen, Pfs230. This finding was observed throughout different stages of gametocyte development. Our findings suggested that the lack of PfEMP1 on gametocyte-IEs may be a key explanation for the low antibody reactivity that contrasts the high reactivity to trophozoite-IEs. We demonstrated that the antibody response to asexual trophozoite-IEs is PfEMP1-dominant, with low levels of antibodies measured to genetically-modified trophozoite-IEs with suppressed PfEMP1 expression. Furthermore, antibody levels to gametocyte-IEs were comparable to trophozoite-IEs that

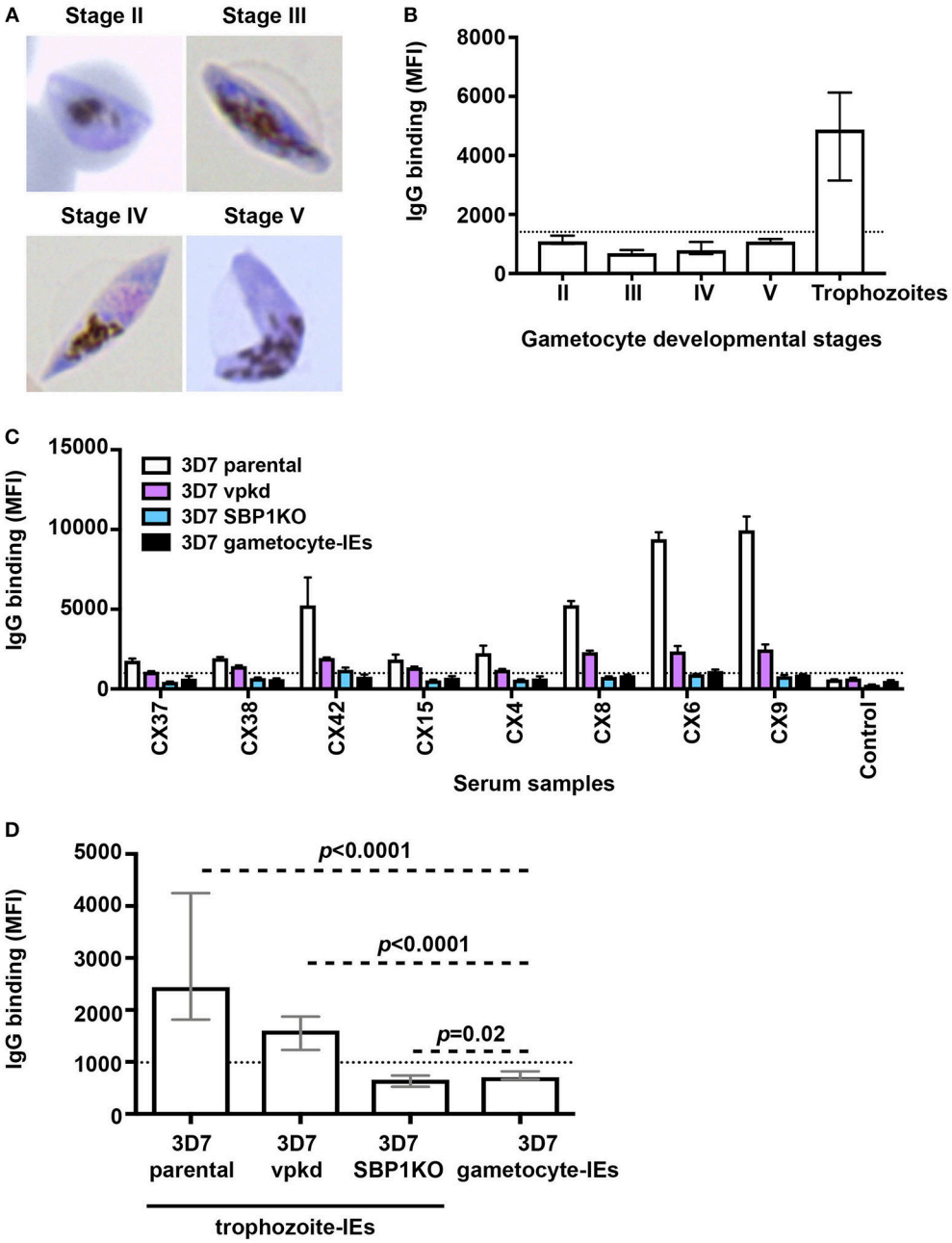

FIGURE 1 | Low levels of naturally-acquired antibodies to the surface of gametocyte-IEs. **(A)** Giemsa-stained smears confirm the respective gametocyte-IE stages. **(B)** Total IgG binding to the surface of trophozoite-IEs and gametocyte-IEs was measured at stages II–V of gametocyte development. Samples were from malaria-exposed Kenyan individuals (children $n = 11$ and adults $n = 10$). The dotted line represents the antibody positivity threshold (MFI levels greater than mean + 3SD of non-exposed Melbourne controls). IgG binding levels are expressed as geometric mean fluorescence intensity (MFI) for all graphs; assays were performed thrice independently, with samples measured in duplicate ($n = 21$); bars represent mean and standard deviation. **(C)** A representative selection of plasma samples tested for antibodies to trophozoite-IEs and gametocyte-IEs. 3D7vpkd and 3D7-SBP1KO are transgenic parasite lines with inhibited PfEMP1 surface expression through the suppression of endogenous *var* genes (var promoter "knock-down"; vpkd) (21, 26) or genetic deletion of the PfEMP1 trafficking protein (skeleton-binding protein 1 "knock-out"; SBP1KO) (22, 27, 28); these were used at the asexual mature trophozoite stage. Samples were from malaria-exposed Kenyan individuals (CX; children $n = 11$ and adults $n = 10$) and non-exposed Melbourne residents (Control). IgG binding to gametocyte-IEs was substantially lower in all individuals compared to IgG binding to trophozoite-IEs. There was minimal background reactivity observed among sera from Melbourne residents; the dotted line represents the antibody positivity threshold. Assays were performed thrice independently; bars represent mean and range of samples tested in duplicate. **(D)** IgG binding to the surface of stage V 3D7 gametocyte-IEs was substantially lower compared to trophozoite-IEs of 3D7 parental and 3D7vpkd. The difference in IgG binding between gametocyte-IEs and trophozoite-IEs of 3D7 SBP1KO was minimal in our sample set. The dotted line represents the antibody positivity threshold (MFI levels greater than mean + 3SD of non-exposed Melbourne controls). Assays were performed thrice independently; bars represent median and interquartile ranges of samples tested in duplicate ($n = 21$; children $n = 11$ and adults $n = 10$); *p*-values were calculated using a paired Wilcoxon signed rank test.

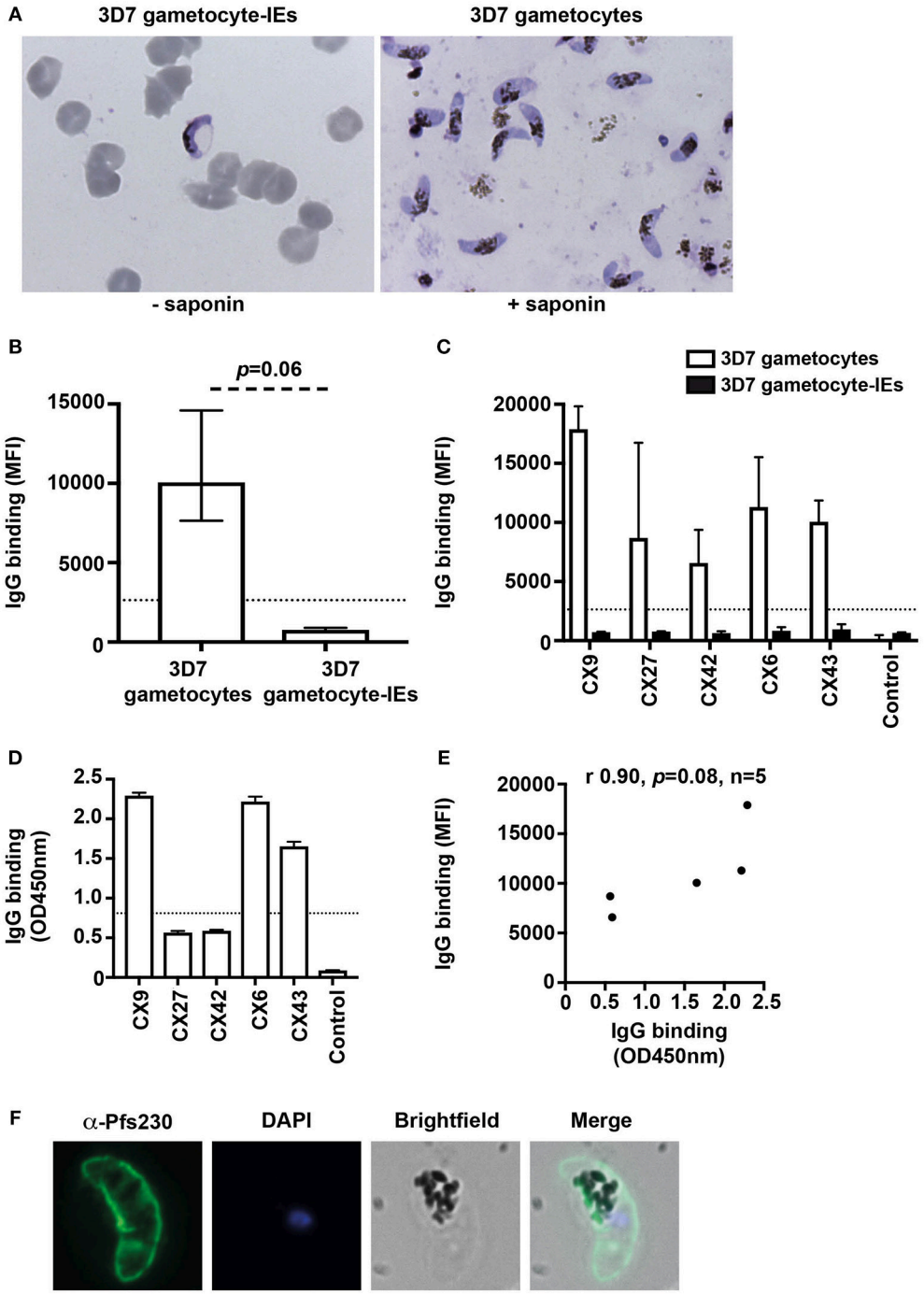

FIGURE 2 | Antibodies recognize the surface of gametocytes in contrast to gametocyte-IEs. **(A)** Giemsa smears of gametocytes (without the erythrocyte membrane) and intact gametocyte-IEs that were used in antibody assays. **(B)** IgG binding to 3D7 gametocytes (without the erythrocyte membrane) was markedly higher compared to intact gametocyte-IEs. Assays were performed thrice independently; bars represent median and interquartile ranges of samples tested in duplicates ($n = 5$; children $n = 3$, adults $n = 2$); p-value was calculated using a paired Wilcoxon signed rank test. The dotted line represents the antibody positivity threshold (MFI levels greater than mean + 3SD of non-exposed Melbourne controls). **(C)** A representative selection of plasma samples tested for antibodies to 3D7 gametocytes and intact gametocyte-IEs. Samples were from malaria-exposed Kenyan individuals (CX; children $n = 3$, adults $n = 2$), and non-exposed Melbourne residents (Control). IgG binding to 3D7 gametocytes was substantially higher in all individuals compared to IgG binding to intact gametocyte-IEs. There was minimal background reactivity observed among sera from Melbourne residents; the dotted line represents the antibody positivity threshold. Assays were performed thrice independently; bars represent mean and range of samples tested in duplicate. **(D)** A representative selection of plasma samples were tested for total IgG binding to recombinant Pfs230D1M. The same selection of samples measured by flow cytometry ($n = 21$; children $n = 11$ and adults $n = 10$) was used. Antibody levels are expressed in

(Continued)

FIGURE 2 | optical density (OD) measured at 450 nm. Assays were performed twice; bars represent mean and range of samples tested in duplicate ($n = 21$); the dotted line represents the antibody positivity threshold. **(E)** There was a strong positive (non-significant) correlation between total IgG binding measured by flow cytometry (MFI) to 3D7 gametocytes and by ELISA (OD450 nm) to recombinant Pfs230D1M. Correlations were evaluated using Spearman's rho (r). **(F)** Immunofluorescence microscopy demonstrates the recognition of the native gametocyte surface by a Pfs230-specific antibody (green). Cells were fixed with 90% acetone and 10% methanol, and DAPI was used to stain nuclear DNA (blue). Representative images are shown.

largely lack PfEMP1. Interestingly, when the IE membrane was removed from stage V gametocytes, acquired antibodies had good reactivity to the gametocyte plasma membrane, indicating that the limited antibody reactivity to gametocyte-IEs was specific to IE surface antigens. Limited antibody acquisition to gametocyte-IEs may be an immune evasion strategy to enable malaria transmission to occur in the face of developing immunity (11, 15, 29, 30). The low or absent expression of PfEMP1 on gametocytes (8) may facilitate this immune evasion given that PfEMP1 is a dominant target of acquired immunity during blood-stage infection (6, 21, 22).

Naturally-acquired antibodies to trophozoite-IEs predominantly target PfEMP1 (21, 22), presented on the IE surface by knob structures (5). However, electron microscopy studies showed that gametocyte-IEs of all stages do not modify the IE surface with knobs, consistent with the absence of the essential knob component, KAHRP (7). Further, *var* gene transcription is downregulated at the onset of gametocyte differentiation, consistent with reports of the absence of detectable surface-exposed PfEMP1 in all stages of gametocyte-IEs (7). The lack of PfEMP1 expression on gametocyte-IEs likely explains the low antibody reactivity observed. As PfEMP1 is the main target of antibodies in asexual trophozoite-IEs, the downregulation of PfEMP1 in gametocyte-IEs may be an immune evasion mechanism to prevent clearance by antibodies. That gametocytes develop within erythrocytes, which lack MHC class-I, may further facilitate immune evasion. Furthermore, gametocyte development occurs predominantly in the bone marrow which may also be a factor influencing the acquisition of antibodies.

Interestingly, when the IE membrane was removed from the gametocytes, human antibodies recognized native antigens expressed on the gametocyte membrane itself, consistent with other reports on permeabilized gametocyte-IEs (13). Major gametocyte surface antigens include Pfs230 and Pfs48/45, which are targeted by human antibodies and associated with transmission-blocking activity through mosquito feeding assays [reviewed in (9)]. We found that the majority of samples tested in our study had high levels of IgG to recombinant Pfs230, suggesting that there was substantial acquisition of antibodies to gametocytes, but not gametocyte-IEs. Further, antibody levels measured to recombinant Pfs230 correlated with those against whole gametocytes.

Prior studies of gametocyte-IEs have reported some acquisition of antibodies mature stage V gametocyte-IEs (11, 12), although the prevalence of these antibodies was generally low. Differences in reactivity to stage V gametocyte-IEs between studies may relate to differences in sample populations and timing of sampling relative to infection episodes, including the presence of active gametocytemias. The focus of our study was to evaluate antibodies to gametocyte-IEs and understand the basis for the different reactivity between trophozoite-IEs and gametocyte-IEs. Future studies are needed to better understand antibodies to mature stage V gametocyte-IEs in different populations (age and geography) with different infection status and exposure history. It is possible that antibodies to gametocyte-IEs could be very short-lived, or antigens expressed could be transient in nature or weakly immunogenic, therefore requiring specific study designs to detect them. Our studies were limited to using the 3D7 *P. falciparum* isolate, and further work to evaluate antibodies to the surface of gametocyte-IEs of other isolates, especially recent clinical isolates, is warranted.

In conclusion, the limited acquisition of antibodies targeting gametocyte-IEs contrasts with the PfEMP1-dominant antibody response toward asexual trophozoite-IEs. Reactivity to gametocyte-IEs was comparable to the low antibody reactivity observed against trophozoite-IEs lacking PfEMP1. However, human antibodies were acquired against the surface of intact gametocytes and to Pfs230. The deficiency in acquired antibodies to gametocyte-IEs could be an important mechanism to avoid clearance by host immunity. Our findings provide new insights to address the major knowledge gaps in understanding immunity and malaria transmission, which will help inform the development of transmission-blocking vaccines.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Alfred Hospital Human Research and Ethics Committee, Australia, Institutional Review Board for Human Investigation at University Hospitals of Cleveland for Case Western Reserve University, USA and the Ethical Review Committee at the Kenya Medical Research Institute with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

J-AC and JB designed the study. J-AC conducted most of the experiments and analyzed the data together with JB. J-AC performed gametocyte culture with guidance from BD and CS. DD produced the recombinant Pfs230D1M protein. LR performed immunofluorescence microscopy. AL-P performed ELISAs. AD, KC, and JK were involved in cohort studies. J-AC,

MB, and JB wrote the manuscript, which was critically reviewed by all authors. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03126/full#supplementary-material

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Differential Patterns of IgG Subclass Responses to *Plasmodium falciparum* Antigens in Relation to Malaria Protection and RTS,S Vaccination

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Naturally acquired immunity (NAI) to *Plasmodium falciparum* malaria is mainly mediated by IgG antibodies but the subclasses, epitope targets and effector functions have not been unequivocally defined. Dissecting the type and specificity of antibody responses mediating NAI is a key step toward developing more effective vaccines to control the disease. We investigated the role of IgG subclasses to malaria antigens in protection against disease and the factors that affect their levels, including vaccination with RTS,S/AS01E. We analyzed plasma and serum samples at baseline and 1 month after primary vaccination with RTS,S or comparator in African children and infants participating in a phase 3 trial in two sites of different malaria transmission intensity: Kintampo in Ghana and Manhiça in Mozambique. We used quantitative suspension array technology (qSAT) to measure IgG_{1-4} responses to 35 *P. falciparum* pre-erythrocytic and blood stage antigens. Our results show that the pattern of IgG response is predominantly IgG1 or IgG3, with lower levels of IgG2 and IgG4. Age, site and RTS,S vaccination significantly

affected antibody subclass levels to different antigens and susceptibility to clinical malaria. Univariable and multivariable analysis showed associations with protection mainly for cytophilic IgG3 levels to selected antigens, followed by IgG1 levels and, unexpectedly, also with IgG4 levels, mainly to antigens that increased upon RTS,S vaccination such as MSP5 and MSP1 block 2, among others. In contrast, IgG2 was associated with malaria risk. Stratified analysis in RTS,S vaccinees pointed to novel associations of IgG4 responses with immunity mainly involving pre-erythrocytic antigens upon RTS,S vaccination. Multi-marker analysis revealed a significant contribution of IgG3 responses to malaria protection and IgG2 responses to malaria risk. We propose that the pattern of cytophilic and non-cytophilic IgG antibodies is antigen-dependent and more complex than initially thought, and that mechanisms of both types of subclasses could be involved in protection. Our data also suggests that RTS,S efficacy is significantly affected by NAI, and indicates that RTS,S vaccination significantly alters NAI.

Keywords: Malaria, *Plasmodium falciparum*, antibody, IgG subclass, naturally acquired immunity, protection, vaccine, children

INTRODUCTION

Malaria caused by *Plasmodium falciparum* is a significant health problem particularly in children under 5 years old in sub-Saharan Africa, with about 219 million cases and 435,000 deaths worldwide (1). In areas of heavy and continuous transmission of *P. falciparum*, naturally-acquired immunity (NAI) to malaria is acquired with age and exposure (2). NAI is mediated by IgG antibodies mainly to antigens of the parasite asexual blood stage (BS) (2, 3) but the specific epitope targets, subclasses and effector functions have not been unequivocally defined. Elucidating these knowledge gaps is important for the development and improvement of vaccines to control and eliminate malaria. Despite the lack of a highly efficacious malaria vaccine, the most advanced product, RTS,S/AS01, is due to start implementation studies in 2019. RTS,S/AS01E has already undergone a phase 3 trial in Africa, where it showed partial protection with overall vaccine efficacy of 25.9% in infants and 36.3% in children (4). RTS,S is a self-assembling virus-like particle consisting of a recombinant protein containing part of the central tandem repeat from the *P. falciparum* circumsporozoite protein (CSP) plus epitopes from the CSP carboxy-terminal, targeting the sporozoite, and liver stages of infection, and fused to the S surface antigen of hepatitis B (HBsAg) virus and coexpressed with HBsAg alone. In the phase 3 trial, RTS,S was formulated in the AS01E liposomal adjuvant containing monophosphoryl lipid A and QS21 and was designed to induce strong anti-CSP antibody and T helper 1 cell responses (4). Although NAI is mainly directed against BS antigens, a natural response to CSP also exists (5) and immunization with irradiated sporozoites confers sterile immunity (6).

Sero-epidemiological studies usually measure total IgG, but the specific subclass is less frequently studied. The relevance of quantifying IgG subclasses relies on their different biological properties. As a consequence, differential associations of each subclass with protection may be masked when studied together. IgG1 and IgG3 are considered to be protective antibodies against *Plasmodium* spp. infection (7–11, 11–15). They are known as the cytophilic subclasses due to their high affinity for most of the Fc receptors on diverse immune cells and their function in complement fixation and opsonization (16). This gives them the ability to mediate protection against malaria through complement-mediated lysis (17) and cell-mediated mechanisms, such as opsonic phagocytosis (15, 18–20) and antibody-dependent cellular inhibition (ADCI) (21). IgG2 and IgG4 have been classically considered as non-protective antibodies against malaria (6–8, 12, 20). In contrast to the previous subclasses, IgG2 and IgG4 have low or no affinity for complement, respectively, and are known as non-cytophilic, because they poorly engage Fc receptors (16). Therefore their main function is neutralization (16). However, IgG4 has high affinity for the activating receptor FcγRI (22), which is expressed on macrophages, monocytes, activated neutrophils, eosinophils and mast cells and is regulated by exposure to cytokines (23). In addition, IgG4 has the highest affinity compared to other subclasses to the inhibitory receptor FcγRIIb (22), at moderate levels present in B cells, macrophages and basophils (23). IgG2 and IgG4 are also suggested to have higher affinity for antigens compared to IgG1 and IgG3. As a consequence, they might out-compete the cytophilic subclasses, preventing or inhibiting cell activation (24, 25).

However, not all reports are consistent with this classical view of protective cytophilic and non-protective non-cytophilic antibodies. For example, IgG2 may have cytophilic properties in individuals carrying the H131 allele of the FcγRIIa receptor, which can bind IgG2 (26). Further, IgG2 has been associated with malaria protection (27), mostly in populations that carry the H131 allele (28, 29). Interestingly, the H131 allele has decreased binding for IgG4 compared to the other allele R131. In addition, anti-CSP IgG4 showed a possible association with protection in RTS,S/AS01 vaccinated subjects (30) and a human IgG4 monoclonal antibody against *P. falciparum* sporozoite inhibited hepatocyte invasion by sporozoites *in vitro* (30). However, our recent phase 3 trial studies with RTS,S/AS01E-induced anti-CSP

antibodies showed that IgG4 to CSP was not associated with protection, whereas IgG1 and IgG3 were (31).

Apart from naturally-acquired IgG responses, maternally transferred antibodies are a source of immunity during the first months of life, and they need to be taken into account when including infants in field studies of malaria immunity, particularly as baseline factors. IgG crosses the placenta, but the subclasses show differences: IgG1 is preferentially transferred, followed by IgG4, IgG3, and IgG2 (16, 32–34). Therefore, the ability of each IgG subclass to cross the placenta might also influence the susceptibility to *Plasmodium* spp in infants and should be taken into account when analyzing IgG patterns in individuals of this age.

We have previously developed and optimized assays (35, 36) to measure IgG, IgG_{1-4} to large panels of diverse antigens with different immunogenicities using the quantitative suspension array technology (qSAT). In the context of the African pediatric multicenter RTS,S/AS01E phase 3 trial for licensure (37, 38), we previously analyzed the role of IgG, $IgG_{1-4,}$ and IgM to RTS,S antigens in vaccine efficacy (31). We found that the pattern of vaccine-induced IgG subclasses for CSP was key in immunity, with IgG1 and IgG3 being protective and IgG2 and IgG4 detrimental. Here, we aimed to better understand the differential role that IgG_{1-4} subclass responses to *P. falciparum* antigens considered as targets of NAI might have in protection against clinical malaria disease in children who are naturally exposed to the parasite and who receive a primary immunization course with three doses of RTS,S/AS01E or a comparator vaccine. To this end, we analyzed at two different time points the levels of IgG subclasses to a range of RTS,S-unrelated antigens in African children from two different malaria endemic areas, evaluating the effect of age, site, and vaccination, and the association of those antibody subclasses with protection against malaria.

MATERIALS AND METHODS

Study Design

This study was carried out in two of the seven sites included in the multicenter immunology study MAL067, ancillary to the phase 3 randomized clinical trial MAL055 (NCT00866619): Kintampo in Ghana (representative of moderate-high malaria transmission intensity [MTI]) and Manhiça in Mozambique (representative of low MTI) (39) to be able to compare the antibody responses in relation to endemicity. These two sites were chosen due to higher availability of sufficient numbers and volumes of samples from both study visits and age cohorts. Subjects were followed up by passive case detection (PCD) starting 14 days after sample collection at month (M) 3, approximately 44 days after the third dose (M2), for the subsequent 12 months, when they were censored.

Children 5-17 months and infants 6–12 weeks at recruitment with $\geq$150 μL plasma/serum samples available at M0 (baseline) and M3 were selected. We included 129 RTS,S/AS01E—(46 infants and 33 children in Manhiça, 26 infants, and 24 children in Kintampo) and 66 comparator—(23 infants and 15 children in Manhiça, 14 infants, and 14 children in Kintampo) vaccinated subjects from both sites (total $n =$ 195). For the correlates of malaria protection and risk analysis, 78 children and infants were randomly selected from Kintampo, and 117 participants were selected from Manhiça according to a prior case-control study of cellular markers (40) and all were analyzed in a case-control design.

The study protocol was approved by the Ethics Committees from Spain, Mozambique and Ghana, and written informed consent was obtained from parents or guardians.

Antibody Assays

qSAT was used to measure antibody responses to 35 *P. falciparum* antigens (**Supplementary Table 1**) applying the xMAP™ technology (Luminex Corp., Texas). Antigens were selected on the basis of profiling BS immunity, but also for effect of vaccination on PE immune responses to sporozoite (SSP2/TRAP and CelTOS) and liver stages (LSA1). Although some of the BS antigens have been characterized as markers of exposure, such as AMA1 and $MSP1_{42}$ (**Supplementary Table 1**), antigen selection was primarily directed toward prominent targets of NAI, vaccine candidates or prior association with protection in sero-epidemiological studies or animal models. Additionally, several antigens were specifically included with said characteristics and limited polymorphism (e.g., Rh2, Rh4, Rh5, and EBA140). VAR2CSA, a pregnancy-specific variant of *P. falciparum* erythrocyte membrane proteins, was included as a representation of maternally-derived antibodies. qSAT assays included bovine serum albumin (BSA) and glutathione S-transferase (GST) coupled beads for background determination and as a control for signal from non-specific binding of *P. falciparum* GST fusion proteins, respectively. *P. falciparum* proteins were covalently coupled directly to MagPlex beads and blocked with BSA. qSAT assays were previously standardized and optimized to control for sources of variability (35, 36, 41). Briefly, antigen-coupled multiplex beads were mixed with 50 μL of test sample, negative or positive control (42, 43), at multiple dilutions (see **Supplementary Materials**). After incubation and washing, biotinylated secondary antibodies were added. Following streptavidin-R-phycoerythrin incubations, samples were acquired with a Luminex 100/200 analyzer and antibody levels measured as median fluorescence intensity (MFI). Data pre-processing is detailed in **Supplementary Materials.**

Statistical Analysis

Comparisons of crude Ig levels across antigens and IgG subclasses were done through boxplots with geometric means, medians and interquartile ranges (IQR), by *t*-tests, and *p*-values adjusted by the Benjamini-Hochberg approach (44) considering all antigens together within each subclass. Analyses included either all subjects or separately by visit and by vaccination, and in some cases stratifying by site, by age, and by age within a site.

To evaluate factors affecting M3 Ig levels to all antigens, we fitted first univariable and next multivariable linear regression models (coefficient, 95% CI, adjusted p-values) using M3 antibody levels (log_{10} MFI) as an outcome and including the following predictors: vaccination, sex, malaria transmission season at M3, having clinical malaria episodes between M0 and M3, and baseline variables like age (in weeks), antibody levels

(log_{10} MFI), hemoglobin (Hb) concentration, weight-for-age Z score (WAZ), and height-for-age Z score (HAZ). Models were also fitted separately at pre-vaccination. Malaria transmission season was defined as high between April-October for Kintampo, and November-April for Manhiça; the remaining months were defined as low transmission. The effect of baseline antibody levels (resulting from prior parasite exposure in the child and/or maternal transfer in the infants) was evaluated using the same antigen/Ig as the outcome variable at M3. The purpose of presenting the analysis of the antibodies at baseline (before vaccination) is that our prior studies showed that they have a very significant influence on the M3 antibody levels and subsequent risk of clinical malaria (31). Linearity of the associations with continuous covariates was evaluated through penalized splines in generalized additive models (GAM); variables were modeled as linear. A stepwise algorithm was used in multivariable models.

Analysis of antibody correlates of protection was based on a case-control design. The outcome was clinical malaria detected by PCD defined by fever >37.5°C with any parasitemia (without a detection limit) in the 12 months after the start of follow-up (M3 plus 14 days). Logistic regression models (odds ratio [OR], 95% CI, adjusted *p*-values) were fitted first univariable and next multivariable to obtain the effect of different predictors in the odds of having malaria. Main predictors included levels (log_{10}MFI) of antibodies at M3, and increment (log_{10}-transformed) of antibody levels between M0 and M3. The impact of the other covariates (same as above) on the association between antibody responses and malaria risk/protection was also assessed. The linearity of the log_{10}-transformed antibody levels was evaluated when the outcome was case-control.

Multivariable models were obtained through the stepwise algorithm, R package MASS (45) and function stepAIC. Both backwards and forward methods were combined to obtain the model with the minimum akaike information criterion (AIC). All potential variables were proposed in the first step of the model, not only the ones significant in univariable analysis. Correction for multiple testing was done by Benjamini-Hochberg. In the above analyses, statistical significance was defined at the level of $p < 0.05$, and trend up to $p = 0.1$.

Finally, we performed multi-marker analysis by principal component analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) using the R packages FactoMineR (46) and DiscriMiner (47), respectively. For the PCA analysis, used to reduce all antibody responses to non-correlated variables that summarize the response, we included the log_{10}-transformed levels of all antigen-subclass pairs at M3 to generate the principal components. We selected the first three principal components that best explained the variance of the data and tested these components on the variables of malaria, vaccination, age and site. For PLS-DA, the response variable was clinical malaria and analysis was performed using the log_{10}-transformed levels of all antigen-subclass pairs at M3. Then, we used the PLS-DA components to run logistic regressions, including also the variables of age, site and vaccine in a multivariable model. Finally, we calculated the AUC performance using the prediction of malaria outcome obtained with the PLS-DA.

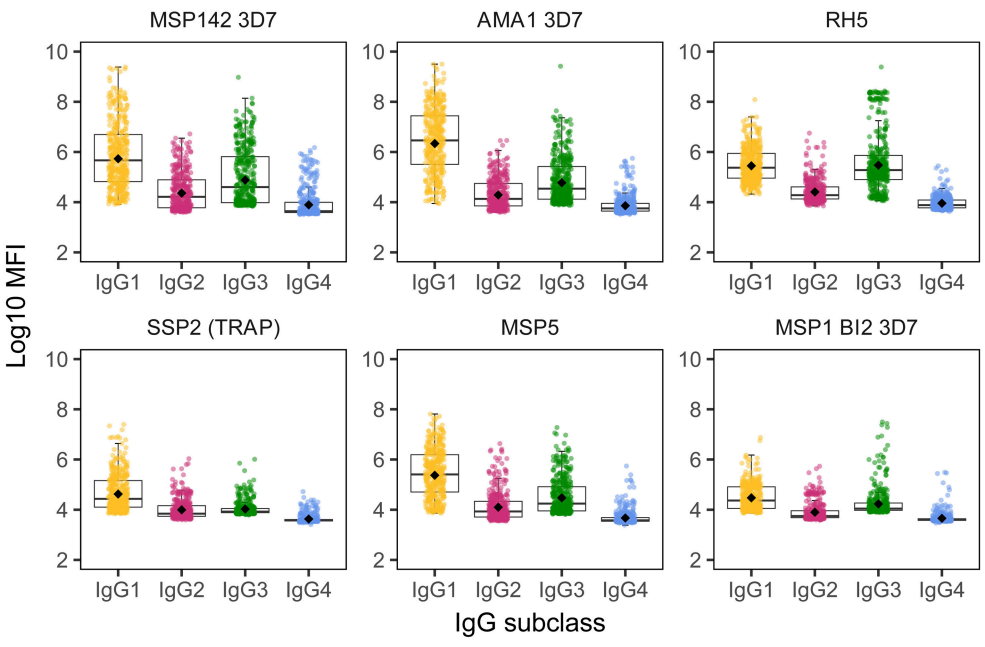

FIGURE 1 | IgG subclass distribution to *Plasmodium falciparum* antigens. Representative examples from the following parasite antigens (**Supplementary Table 1** for more details) whose analysis of antibody subclass responses showed differential patterns: $MSP1_{42}$ (3D7 strain), AMA1 (3D7 strain), RH5, SSP2 (or TRAP), MSP5, and MSP1 block 2 (3D7 strain).

RESULTS

Baseline Characteristics

RTS,S and comparator vaccinees were similar with regards to baseline characteristics (age, sex, weight, height, other vaccinations, previous malaria, season, distance to health center, Hb concentration) and most of them (93%) completed the 12-month post-vaccination follow-up (31). The median time to drop out from the study was 113 (range 21–276) days, and was due mostly to early terminations due to loss to follow-up (7 subjects) or migration (4 subjects). A total of 89 malaria clinical events were recorded during the follow-up time: 60 in Kintampo (36 in RTS,S and 24 in comparators) and 29 in Manhiça (18 in RTS,S and 11 in comparators). Thirty-five clinical malaria events (39%) were registered in the children age cohort (48% in Kintampo [$n = 29$], 21% in Manhiça [$n = 6$]), and the remaining in the infant age cohort. Parasitemia of subjects who had clinical malaria was comparable between RTS,S and comparator vaccinees. The Kaplan Meier median follow-up time was 365 days (IQR = 128 to 365).

Factors Affecting Levels of IgG Subclasses to *P. falciparum* Antigens

We detected IgG responses to most malarial antigens with a predominance of the IgG1 subclass, followed by IgG3, although some antigens (MSP2, MSP1 block [bl] 2 constructs, except the Well strain, and RH5) had similar levels of IgG1 and IgG3 (**Figure 1** and **Supplementary Figure 1)**. Levels of IgG2 were lower than those of IgG1 or IgG3, while levels of IgG4 were the lowest. The magnitude of responses at the study visits M0 and M3 (1 month post-vaccination) was differentially affected by several factors.

Effect of RTS,S Vaccination

For IgG1, three different patterns of antibody response to vaccine-unrelated antigens emerged at M3 upon RTS,S immunization compared to M0 or with comparator vaccinees (**Figures 2**, **3** and **Supplementary Figure 2,** multivariable models): decrease in antibody levels, increase in levels, and no change in levels. In multivariable models, RTS,S vaccination decreased significantly IgG1 levels to $MSP1_{42}$ 3D7, AMA1 FVO, and pTRAMP (**Figure 3**). In contrast, RTS,S vaccination significantly increased IgG1 levels to DBLα, MSP1 bl2 (3D7, Well, RO33, and Mad20 strains), MSP6, RH2 2030, EBA175 R3-5, MSP5, EBA140 R3-5, and RH4.2 (trend for MSP2 CH150 and RH5). The IgG1 levels to the rest of antigens were not significantly affected by RTS,S vaccination. IgG3 antibody levels to DBLα, SSP2, MSP5, and MSP1 bl2 (Well and RO33 strains) were higher after RTS,S vaccination (trend for RH2 2030 and RH4.2), but the rest of responses did not change. IgG2 levels to EXP1 decreased after RTS,S vaccination while levels increased for VAR2CSA DBL3-4 and MSP5. The levels of IgG4 did not significantly change with vaccination except for MSP5 that were increased as with the other subclasses. PCA analysis (**Figure 4A**) showed that, globally, IgG subclass responses did not differ significantly by vaccination.

Effect of Age

Comparing antibody profiles over time, IgG levels significantly decreased from M0 to M3 for some antigens probably reflecting the decay of maternal antibodies, while for others there was a statistically significant increase and others did not show differences (**Supplementary Figure 3**). Notably, for most antigens, the increase was mainly observed in RTS,S vaccinees, confirming the effect of vaccination described above. In multivariable analysis, the effect of age group on baseline levels of IgG1 and IgG3 had a mixed pattern. Levels against 11 of 35 antigens were significantly higher in infants than children, while levels against 10 and 13 out of 35 antigens were significantly higher in children than infants for IgG1 and IgG3, respectively (**Figure 5**). These latter antigens were pTRAMP, RH1, MSP3 3C, CyRPA, CelTOS, RH4.9, and MSP5 for both subclasses, RH5, EBA140, MSP1 bl2 Well for IgG1, and p41, RH2 b240, LSA1, DBLα, and SSP2 for IgG3. Levels of IgG2 were generally lower in children than infants (12 out of 35 antigens) or not different except for MSP5 and MSP2 bl2 Mad20, for which IgG2 levels were significantly higher in children than infants (**Figure 5**). In contrast, IgG4 levels to 17 of 35 antigens were significantly higher in children than infants, except for $MSP1_{42}$ 3D7, EXP1, and MSP2 for which levels were significantly lower in children. In multivariable models adjusted by M0 IgG levels and vaccination, among other variables, M3 IgG levels were generally higher in children than infants or not significantly different, except for IgG1 to RH4.9 and IgG1 and IgG2 to VAR2CSA DBL3-4 that were lower (**Figure 3**). These M0 and M3 results could be observed also in univariable analyses (**Supplementary Figure 4**). The higher baseline IgG1 and IgG3 levels against many antigens in infants than children suggest maternally-transferred antibodies, whereas IgG4 antibodies appeared to be poorly transferred and/or already induced in the first months of life. Higher levels in children than infants at M3 adjusting for baseline levels suggests higher acquired responses in children than infants during the 3-month period. Analysis of IgG responses by PCA at M3 (**Figure 4B**) showed that infants clustered together while children were more disperse. The variables that contributed the most to PC1 and PC2 and contributed to segregate partially the responses that corresponded to children and infants were, on one hand, IgG3 to $MSP1_{42}$ 3D7 and, on the other hand, IgG3 to MSP1 bl2 Mad20, and IgG4 to RH2 b240, LSA1, CelTOS, SSP2, RH2 (2030), MSP1 bl2 Mad20, EBA140 R3-5, and RH4.2.

Effect of Site (MTI)

In general, antibody levels were higher in Kintampo (high MTI) than in Manhiça (low MTI) for most antigens (**Supplementary Figure 5**). At M0, antibody levels were significantly higher in Kintampo than in Manhiça to most antigens except for IgG2 to MSP1 bl2 Mad20 and IgG4 to MSP3 3C, RH2 b240, LSA1, MSP1 bl2 (PA17 and Mad20) CelTOS, SSP2, and MSP5, which were significantly higher in Manhiça than Kintampo (**Figure 5** and **Supplementary Figures 6**, **7**). In contrast, at M3 a more mixed pattern was observed in models adjusted by baseline levels and vaccination, among other variables. Some responses did not significantly differ by site

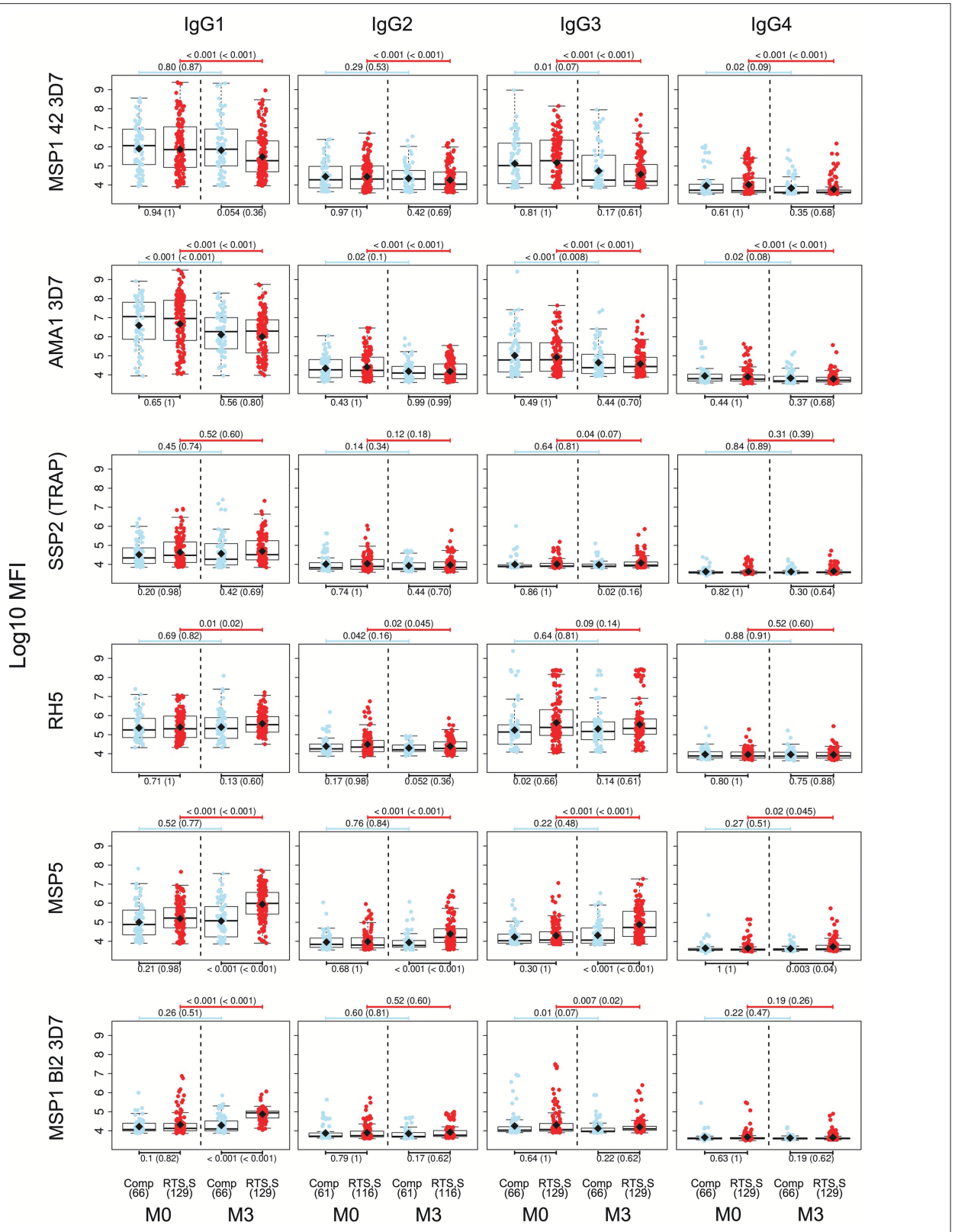

FIGURE 2 | IgG subclass distribution to *Plasmodium falciparum* antigens per visit and vaccination group. Representative examples. Adjusted *p*-values are shown in parenthesis.

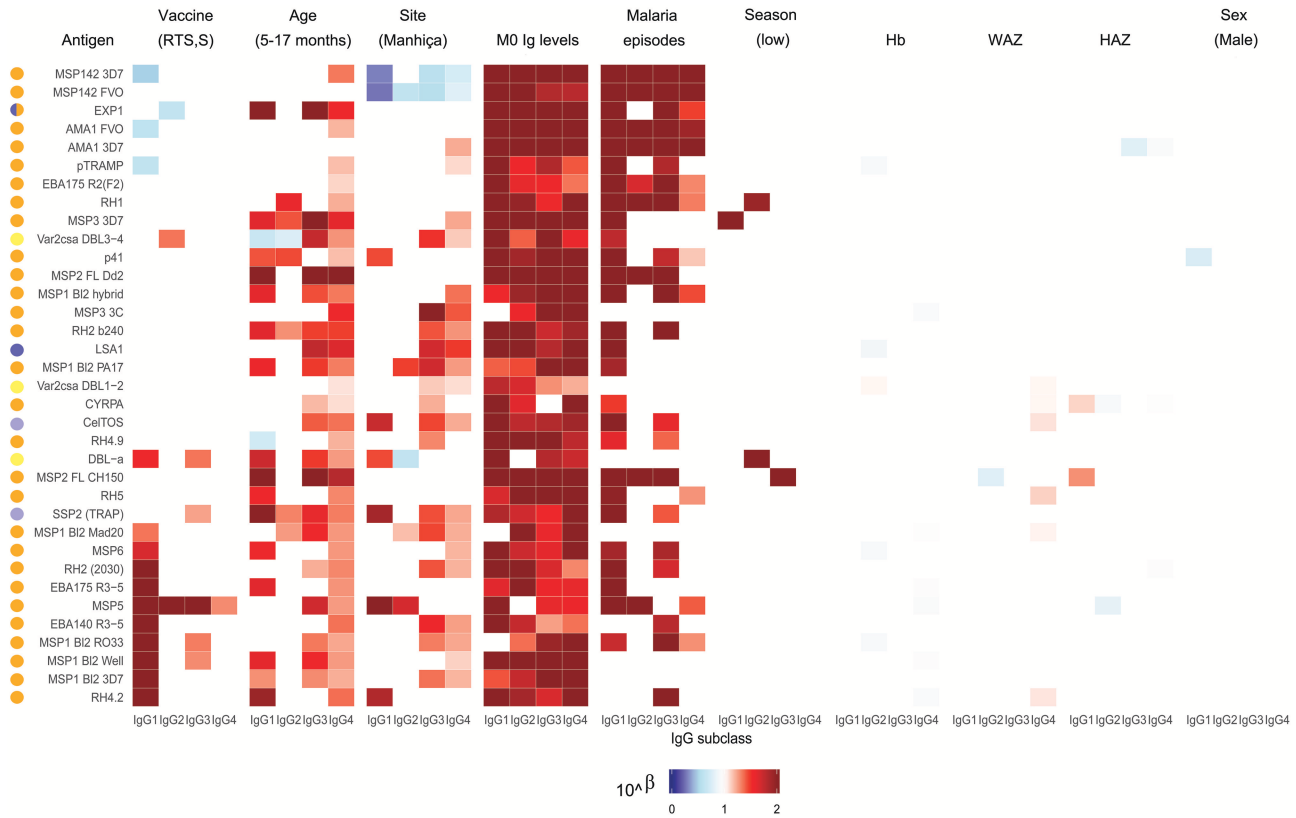

FIGURE 3 | Heatmap of multivariable analysis of the effect of RTS,S/AS01E vaccination and demographic, clinical, and epidemiological variables on IgG subclass levels to *Plasmodium falciparum* pre-erythrocytic and blood stage antigens at month 3 visit. Linear regression was performed with antibody levels (log_{10}MFI) as outcome and other covariates adjusted (in parenthesis the reference category). Color coded cells show the 10^Beta of each predictor (columns) for each model (rows). Only results with a significant (<0.05) *p*-value are shown. Antigen colors show the *P. falciparum* life cycle stage in which they are expressed: Pre-erythrocytic (PE) antigens from sporozoite stage (light purple), PE from liver stage (dark purple), blood stage (BS) antigens from merozoite stage (orange), BS from trophozoite stage (yellow).

(27/35 antigens for IgG1 and 30/35 antigens for IgG2), others were higher in Manhiça than Kintampo (6/35 antigens for IgG1, 3/35 for IgG2, 15/35 for IgG3 and 19/35 for IgG4), and only anti-$MSP1_{42}$ antibodies of all subclasses were lower in Manhiça than Kintampo (**Figure 3** and **Supplementary Figures 6**, **7**). Thus, children and infants in Kintampo had significantly higher levels to all subclasses and antigens with the exception of IgG4 against some antigens, which were significantly higher in Manhiça, but Manhiça subjects had increased levels of the other subclasses from M0 to M3 to similar or higher levels than Kintampo subjects. Analysis by PCA at M3 (**Figure 4C**) showed clustering of individuals by site, although there was some overlap with age. Most infants from both sites clustered together whereas PC2 discriminated children from each site (**Figure 4D**).

Effect of Other Variables

At baseline there were not very generalized or consistent significant associations with antibody levels and the rest of covariates in multivariable models (**Figure 5**). Hb concentrations were negatively associated with IgG1, IgG2, and IgG3 levels to some antigens but positively associated with IgG4 levels. For WAZ there were generally significant positive associations while for HAZ there were significant negative associations, markedly for IgG4. Sex was not significantly associated with M0 levels except for IgG3 to MSP1 bl2 RO33 and Mad20 (females > males) and VAR2CSA DBL1-2 (males > females). At M3, the strongest and more consistent associations were that higher M0 antibody levels and prior malaria episodes were significantly associated with higher M3 antibody levels (**Figure 3**).

Antibody Correlates of Malaria Protection

Baseline antibody levels of most subclasses/antigens were generally significantly higher in those subjects who had clinical malaria during the 12-month follow up after M3 than in those who did not (**Figure 6** and **Supplementary Figure 8**). At M3, IgG1, IgG2 and IgG3 antibodies were also higher in malaria cases for some antigens whose levels decreased from M0 to M3 (e.g., AMA1, $MSP1_{42}$). Stratified by age, this was more significant in children (**Supplementary Figure 9**). Stratified by site, these differences were not detected (**Supplementary Figure 10**). In contrast, M3 IgG1, IgG2, and IgG3 levels that increased after RTS,S vaccination or to those antigens whose antibodies did

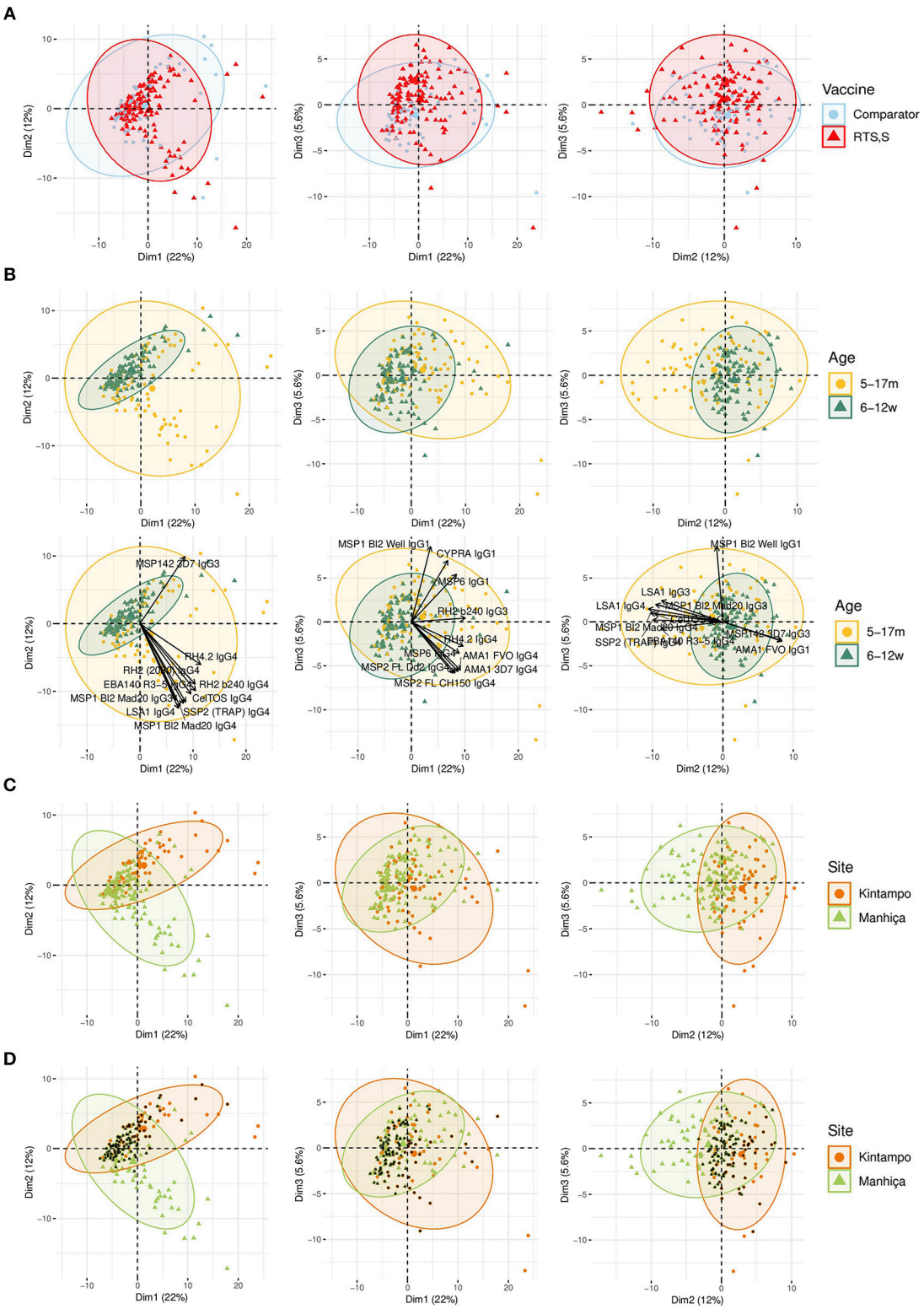

FIGURE 4 | Principal Components Analysis (PCA) of the antibody responses at month 3 (M3). The plots of the distribution of individuals by type of vaccine received **(A)**, age **(B)**, site **(C)**, or site plus age **(D)** are shown. The first three principal components (Dim 1, Dim 2, and Dim 3) that explained the highest percentage of the variance of the antibody responses at M3 (percentage in parenthesis) were chosen for representation. Representation of the 10 first variables (antigen-IgG subclass pairs) that contributed to the principal components **(B)**. Black dots represent infants **(D)**.

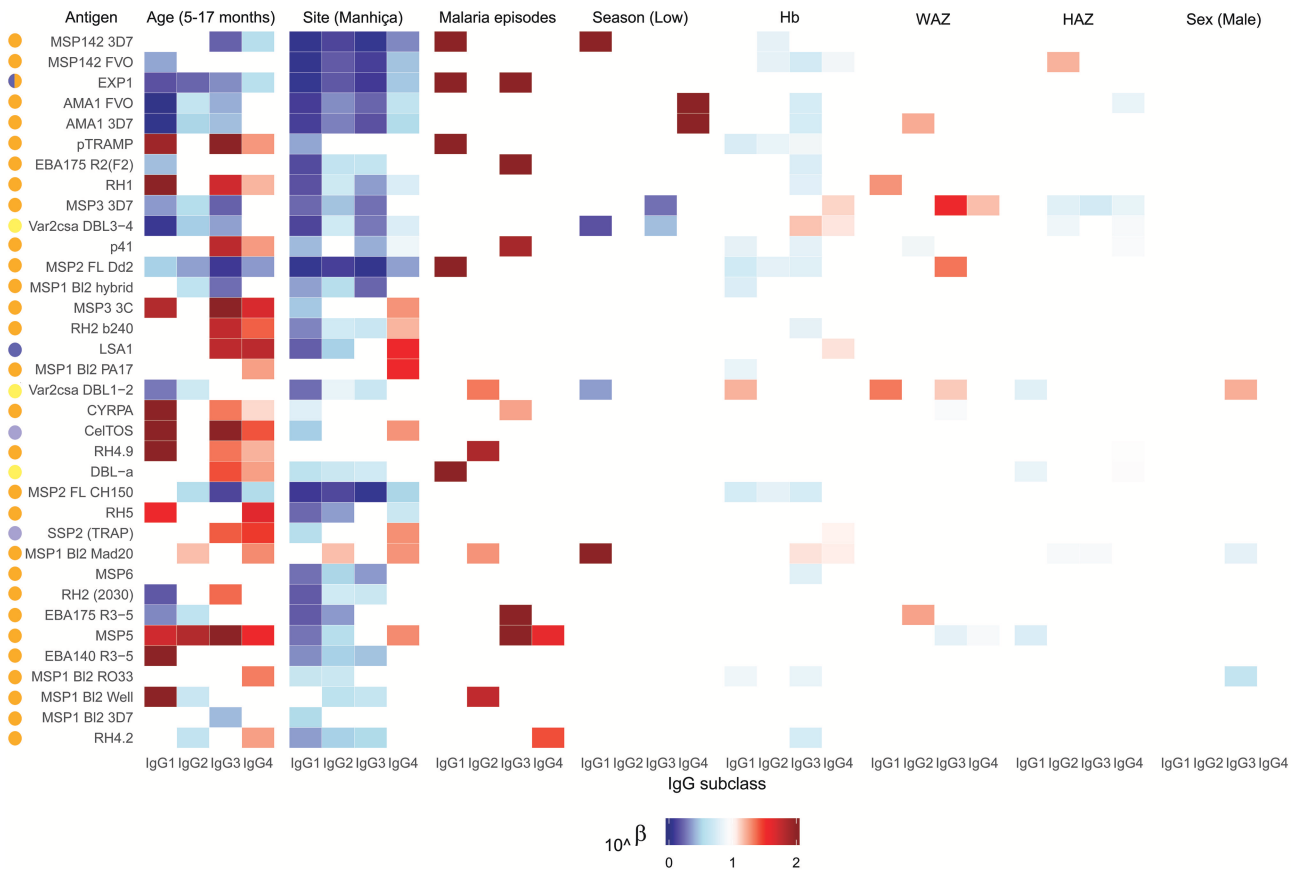

FIGURE 5 | Heatmap of multivariable analysis of the effect of RTS,S/AS01E vaccination and demographic, clinical, and epidemiological variables on IgG subclass levels to *Plasmodium falciparum* pre-erythrocytic and blood stage antigens at baseline. Linear regression was performed with antibody levels (log_{10}MFI) as outcome and other covariates adjusted (in parenthesis the reference category). Color coded cells show the 10^Beta of each predictor (columns) for each model (rows). Only results with a significant (<0.05) *p*-value are shown. Antigen colors show the *P. falciparum* life cycle stage in which they are expressed: Pre-erythrocytic (PE) antigens from sporozoite stage (light purple), PE from liver stage (dark purple), blood stage (BS) antigens from merozoite stage (orange), BS from trophozoite stage (yellow).

not decrease from M0 to M3, did not generally differ in cases and controls (**Figure 6** and **Supplementary Figures 8–10**). IgG4 followed a different pattern; crude levels of antibodies to pre-erythrocytic (PE) antigens SSP2, LSA1, and CelTOS and to MSP1 bl2 constructs were higher in protected than non-protected subjects, not only at M3 but also at M0 (**Figure 6** and **Supplementary Figures 8**). M3 IgG3 levels to these antigens had a similar pattern but associations were weaker. This IgG4 pattern was mainly observed in children (**Supplementary Figure 9**) and in Manhiça (**Supplementary Figure 10**). Stratified by age and site, M3 crude IgG4 (and IgG3) levels to the above and additional antigens were lower in Manhiça children with malaria after RTS,S vaccination than non-malaria controls, while the opposite pattern was seen for comparator vaccinees (**Supplementary Figure 11**), suggesting an interaction with vaccination (evident in this particular group).

Analysis of the change in IgG1 and IgG3 levels from M0 to M3 revealed significantly higher increments in controls than malaria cases to most of the antigens that had increased antibody responses in RTS,S vaccinees, particularly MSP1 bl2 constructs and MSP5 (**Supplementary Figure 12A**). Stratifying by age and site, the association was mostly seen for children and Kintampo (**Supplementary Figure 12B**).

In logistic regression models adjusted by vaccine, age, site and M0, among other variables when relevant, M3 IgG1 to AMA1 and RH1, IgG2 to AMA1 3D7, RH1 and RH5, and IgG3 and IgG4 to MSP2 FL CH150, were associated with increased malaria risk (**Figure 7**). On the contrary, levels of M3 IgG3 to MSP1 bl2 3D7, PA17, and hybrid, VAR2CSA DBL1-2 and LSA1, and IgG4 to MSP1 bl2 PA17 and SSP2, were associated with malaria protection. Also, in multivariable logistic regression models, the difference between M3 and M0 levels for IgG1 to MSP5, MSP6, MSP1 bl2 Well and MSP2 FL Dd2, and IgG3 to VAR2CSA DBL1-2 and MSP5, was associated with protection, while IgG1 to MSP1 bl2 Mad20 was associated with risk (**Figure 7**). In stratified analysis including only RTS,S vaccinees, IgG3 and IgG4 levels to the PE antigens LSA1, SSP2 and CelTOS and MSP1 bl2 constructs were associated with protection. Among covariates retained in multivariable models (**Figure 7**), RTS,S vaccination, children cohort (mostly), Manhiça site (always), low

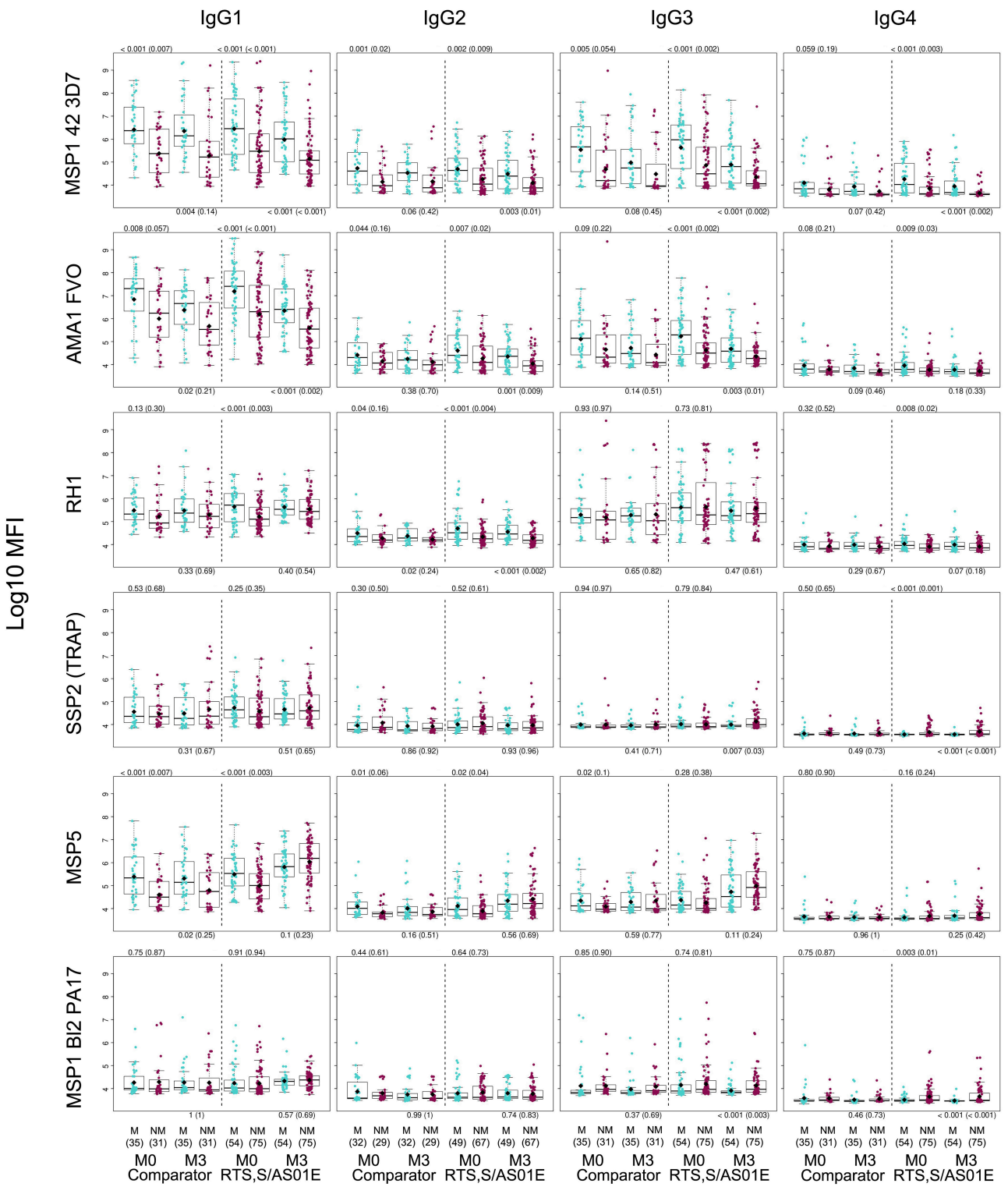

FIGURE 6 | Association between month 3 IgG levels and malaria protection after RTS,S vaccination. Only representative examples are included. Adjusted p-values are shown in parenthesis. M, Malaria, NM, No malaria.

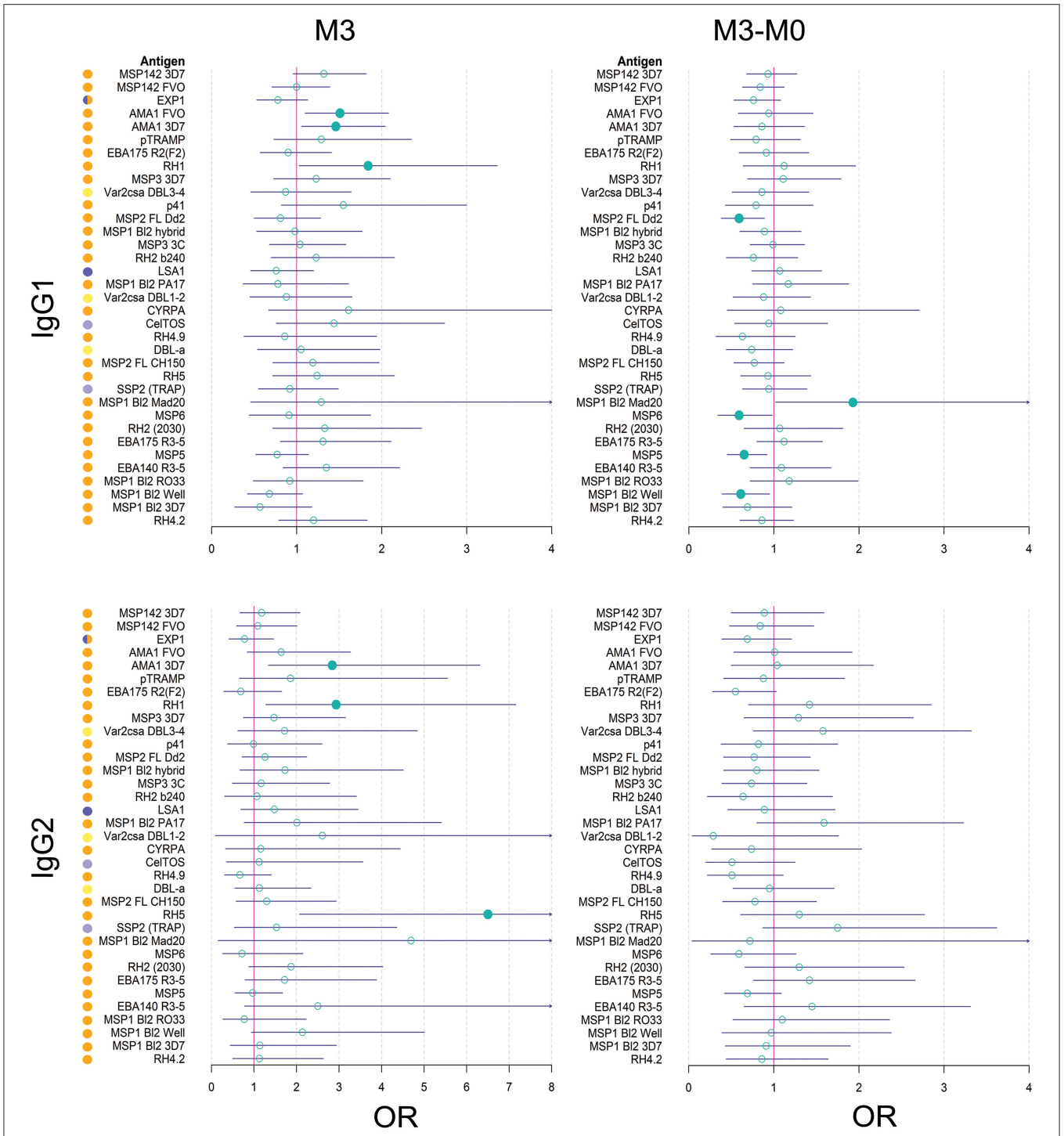

FIGURE 7 | Continued

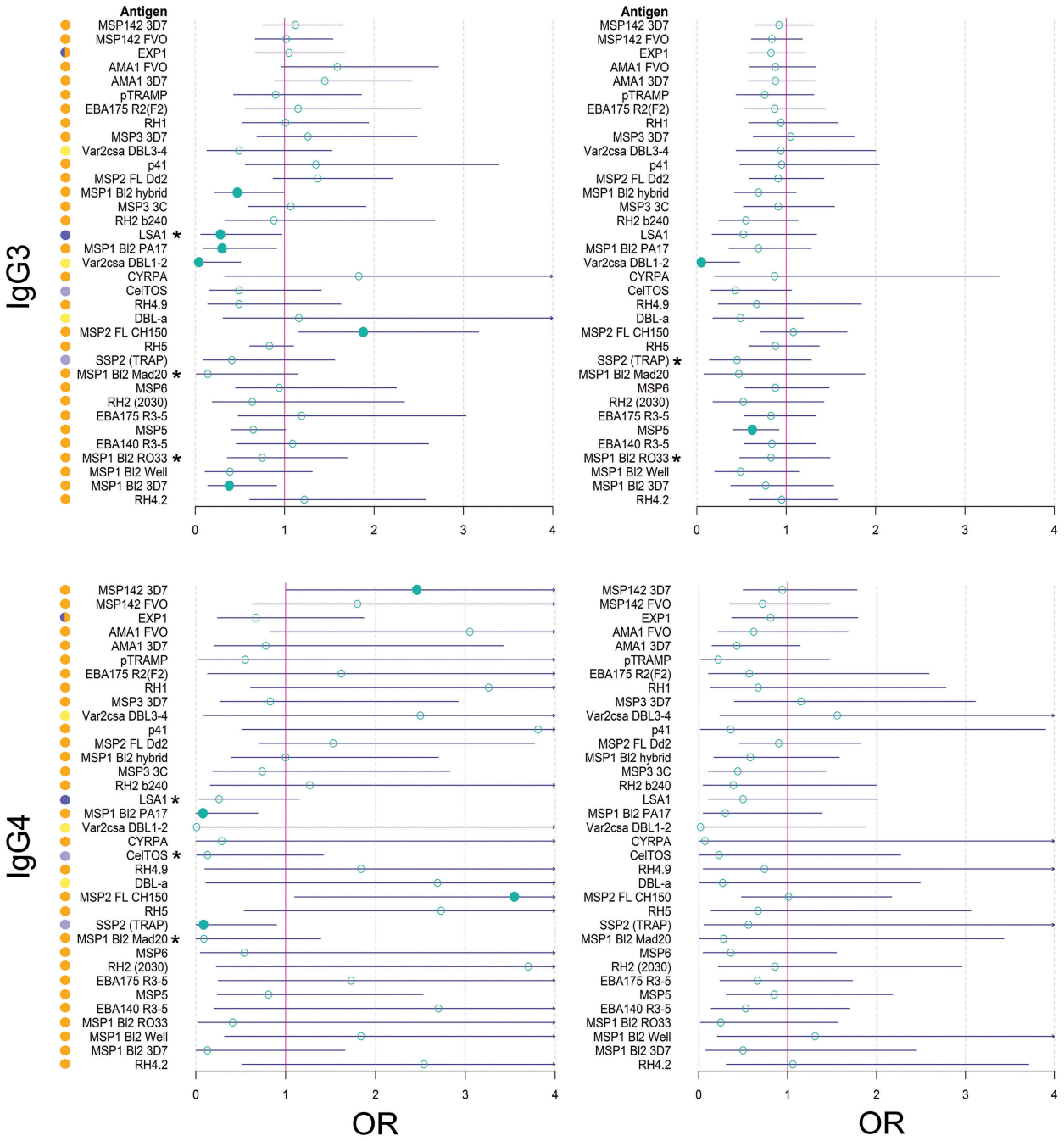

FIGURE 7 | Forest plots of multivariable analysis of the association between IgG subclass responses to *Plasmodium falciparum* pre-erythrocytic and blood stage antigens, and malaria protection. Logistic regression models with antibody levels at month 3 (M3), or change in M3 and M0 levels (M3-M0), as predictors and clinical malaria as outcome, adjusted by covariates that entered the models according to the minimum akaike information criterion (RTS,S vaccination, age cohort, site, M0 antibody levels, WAZ and prior malaria episodes, depending on the antigen/subclass). OR: Odds ratio with 95% confidence intervals (CI). OR with a CI below (protection) or above (risk) 1 are represented by a colored circle. Associations with a CI below or above 1 (asterisks) in stratified analysis including only RTS,S vaccinees: IgG3 to LSA1 at M3: *OR* = 0.13 (0.02;0.71), $p = 0.03$; IgG3 to MSP1 bl2 Mad20 at M3: *OR* = 0.01 (0;0.33), $p = 0.02$; IgG3 to MSP1 bl2 RO33 at M3: *OR* = 0.33 (0.09;0.99), $p = 0.056$; IgG3 to SSP2 (TRAP) at M3-M0: *OR* = 0.23 (0.05;0.86), $p = 0.04$; IgG3 MSP1 bl2 RO33 at M3-M0: 0.47 (0.2;0.99), $p = 0.053$; IgG4 LSA1 at M3: *OR* = 0.07 (0;0.65), $p = 0.058$; IgG4 to CelTOS at M3: *OR* = 0 (0;0.28), $p = 0.03$; IgG4 to MSP1 bl2 Mad20 at M3: *OR* = 0 (0;0.25), $p = 0.055$.

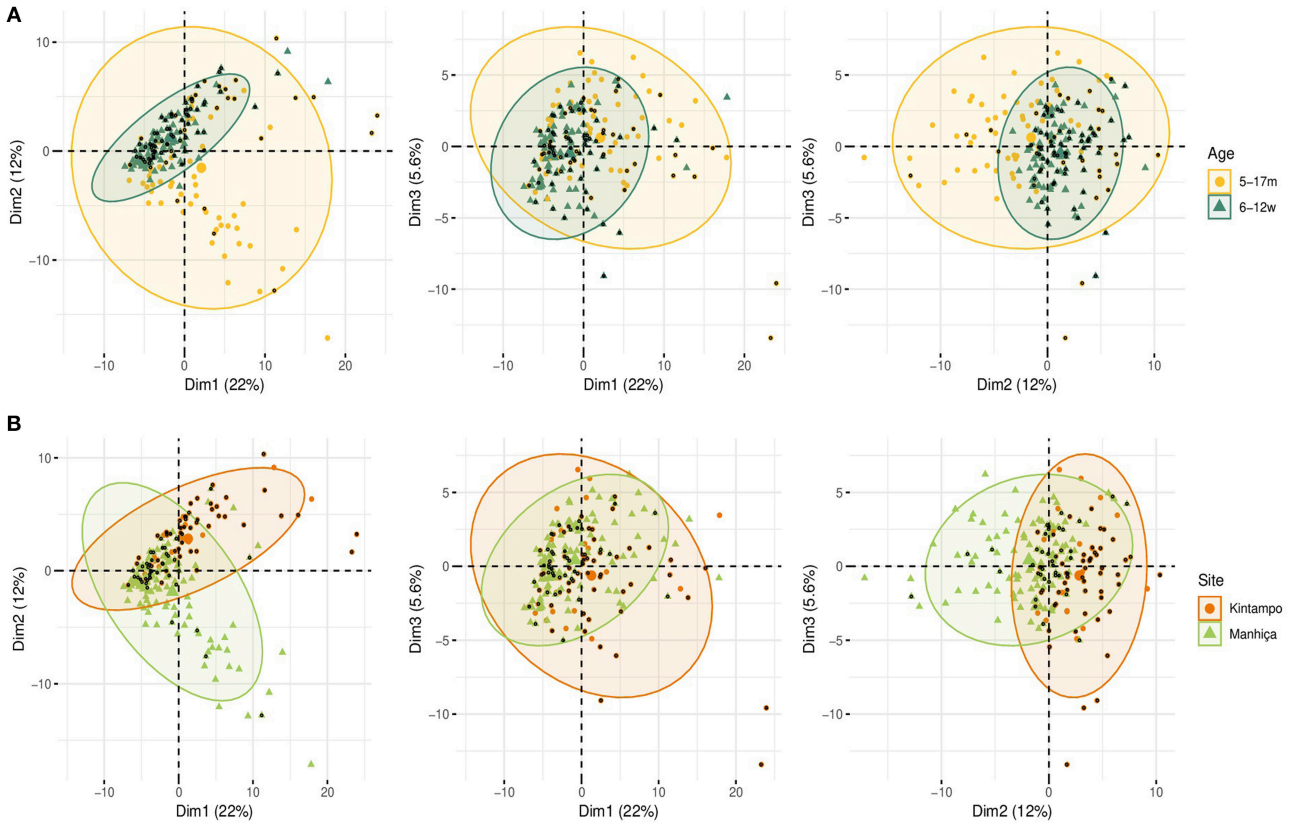

FIGURE 8 | Principal Components Analysis (PCA) of the antibody responses at month 3 (M3). The plots of the distribution of individuals by age **(A)** and site **(B)** plus malaria are shown. The first three principal components (Dim 1, Dim 2, and Dim 3) that explained the highest percentage of the variance of the antibody data at M3 (percentage in parenthesis) were chosen for representation. Black dots represent malaria cases.

baseline antibody levels and high WAZ (specially for IgG2), were associated (statistically significantly or not) with less risk of malaria. Sex, prior malaria episodes, season, HAZ or Hb were not significantly contributors to the models, except for previous malaria that improved the IgG3 model (M3 levels, RTS,S vaccinees only) for MSP1 bl 2 constructs.

In PCA analysis at M3, simultaneous representation of malaria cases by age and site (**Figures 8A,B**) showed that most malaria cases overlapped with infants and Kintampo, respectively. This is in agreement with the observation in multivariable logistic regression models, where children and Manhiça were associated with less risk of malaria.

PLS-DA at M3 with malaria as a response variable (**Figure 9A**) identified three main components associated with malaria. The IgG subclass responses that contributed more to these components (the 10 responses with higher loadings in each component) included IgG1 to $MSP1_{42}$ and MSP2; IgG2 to AMA1, MSP2, RH5, MSP1 bl2 constructs, LSA1, VAR2CSA DBL1-2, RH1; IgG3 to MSP2, pTRAMP, VAR2CSA DBL1-2, CelTOS, RH4.9, MSP5, RH2 (2030), SSP2 and MSP1 bl2 constructs; and IgG4 to MSP1, VAR2CSA DBL1-2 and DBLα (**Figure 9B**). In a multivariable logistic model including these three PLS-DA components and adjusted by age, site and vaccine, the PLS-DA components were independently associated with malaria (component 1 [$p < 0.001$], component 2 [$p = 0.01$] and component 3 [$p < 0.001$]), while age ($p = 0.76$), site ($p = 0.29$), and vaccine ($p = 0.57$) were not. The predictive ability of the model was very high (75 out of 79 malaria cases and 94 out of 96 controls correctly predicted) with an AUC of 0.964.

A summary of all results according to single-marker (univariable and multivariable regression models) and multi-marker (PLS-DA) analysis, is presented in **Figure 10**. This shows the groups of antigens whose levels of antibodies at M3 and/or changes in levels from M0 to M3, were associated with malaria protection (green) or risk (purple), for each IgG_{1-4} subclass response.

DISCUSSION

Our study found that malaria protective immunity is mostly associated with the cytophilic subclasses IgG1 and IgG3 and, in spite of the low levels, with IgG4, to certain PE and BS *P. falciparum* antigens, whereas IgG2 responses were mostly associated with malaria risk. As expected, IgG1 followed by IgG3 were the predominant subclasses in most antigens, with lower IgG2 and IgG4 levels. Consistent with previous reports,

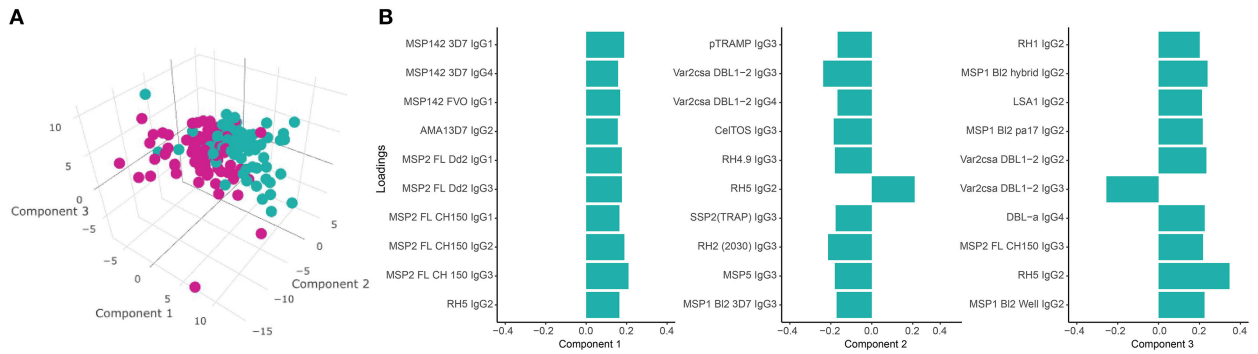

FIGURE 9 | Partial Least Squares Discriminant Analysis (PLS-DA) plots. **(A)** 3D scatter-plot with the three main PLS-DA components that best enable discrimination between malaria cases (M) and non-malaria controls (NM) using antibody levels at month 3 (M3) (cumulative $R^2 = 0.37$). **(B)** Loadings of antigen-subclass pairs with the 10 highest absolute scores for each PLS-DA component at M3.

MSP1 bl2, MSP2, and RH5 predominantly induced IgG3, or similarly high IgG3 and IgG1 levels, and we found that this was also the case for DBLα (48–51). Each IgG subclass and antigen pairs studied showed a different pattern, magnitude, and association with malaria protection, with different factors affecting the outcome. Despite this complexity, we found novel and consistent results regarding determinants of the antibody response and impact on malaria outcomes.

IgG1 and IgG3 were differently associated with malaria protection, depending on the antigen specificity. IgG1 levels to antigens that are considered markers of exposure ($MSP1_{42}$, AMA1, EXP1, etc.) were higher in infants than children and in malaria cases than controls. This could be explained by presence of maternal antibodies, which are preferentially of the IgG1 subclass due to the differential placental transfer (16, 32, 33)—as shown by VAR2CSA antibodies that should be transferred by the mother- and/or a higher pre-exposure to the parasite in these individuals. It has been previously established that a strong risk factor for future malaria is having had malaria in the past (13) which can be manifested by higher baseline levels of IgG1 to these BS antigens (52). In contrast, change from M0 to M3 in IgG1 levels to a different group of antigens (MSP5, MSP6, RH4.2, MSP1 bl2, etc.) was associated with protective responses. In the case of IgG3, levels of antibodies to SSP2, LSA1, and MSP1 bl2 at M3 were associated with less malaria risk.

There are differences between IgG1 and IgG3 that might have implications for protection and that could explain why they differ in levels, even though they show similar antigen reactivity. In terms of similarities, they both respond mainly to proteinaceous antigens and are cytophilic. However, IgG3 has a shorter half-life (7 days) compared to the other subclasses (21 days) (16, 25), meaning that the presence of IgG3 could depend on a continuous or repeated exposure to compensate for the shorter half-life, although some IgG3 allotypes have also shown extended half-lives. Therefore, malaria exposure may influence IgG subclass balance, such that early in infection an IgG1 response may be dominant, and for some antigens this may then evolve to IgG3. In addition, it is suggested that IgG3 has lower antigen-affinity than IgG1, but it activates complement more effectively and has higher affinity to Fcγ receptors (16, 25), thus, when present, it is probably more effective in mediating effector mechanisms.

We hypothesized that while cytophilic antibodies IgG1 and IgG3 would be associated with protection, non-cytophilic antibodies IgG2 and IgG4 would be associated with risk, as reported in other studies (7–11, 11–15, 53), although some studies have found protective associations with IgG2 (54). Unexpectedly, IgG4 showed potential associations with immunity despite presenting the lowest levels. In univariable analysis we found that higher IgG4 levels to all PE antigens and MSP1 bl2 constructs correlated with malaria protection. Interestingly, these responses helped discriminate between children from Manhiça and infants and children from Kintampo, suggesting that these responses may be particularly acquired in Mozambique. In multivariable analysis, higher IgG4 levels to the PE antigens SSP2 and LSA1 and one of the MSP1 bl2 constructs were associated with protection. Regarding IgG2, responses to AMA1, RH1 (like IgG1) and RH5, positively correlated with malaria risk. Consistently, in multi-marker analysis IgG2 to AMA1, RH1, and RH5 were identified as significant variables predicting malaria disease, in addition to MSP2 FL CH150, LSA1, VAR2CSA DBL1-2, and MSP1 bl2 constructs. This is in agreement with the notion that production of non-cytophilic IgG2 elicited by natural exposure to the parasite is non-protective against malaria (7–9, 13, 53). Consistent with this, we have previously found that higher levels of IgG2 to CSP induced by RTS,S vaccination are associated with malaria risk in the phase 3 trial (31). Interestingly, infants had higher IgG2 levels than children (except for MSP5 and MSP1 bl2 Mad20) at baseline, which is surprising because this subclass is considered to be the least efficiently transferred during pregnancy (16, 32, 33). This probably reflects very high maternal IgG2 levels, as shown for VAR2CSA antibodies.

Cytophilic subclasses are well known for mediating protection in malaria through their ability to fix complement (17) and mediate opsonic phagocytosis (15, 18–20) and ADCI (21). A previous observation that phagocytic activity was lower in protected than non-protected subjects vaccinated with RTS,S/AS01 (55) led to the discovery that the neutralizing and/or

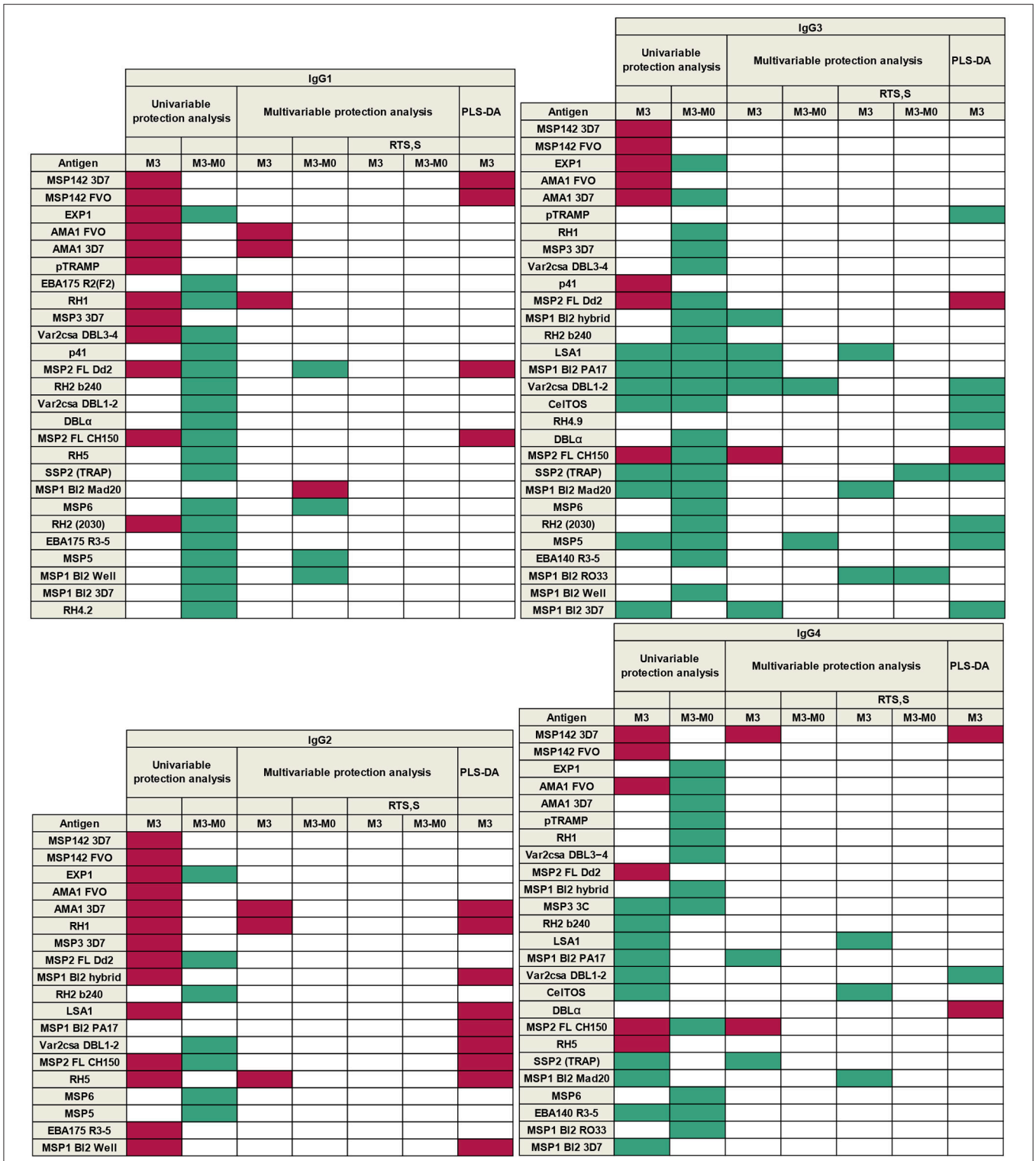

FIGURE 10 | Summary of associations between antibody responses and malaria protection. Purple represents association with malaria risk and green with protection.

inhibitory function of IgG4 to CSP is associated with reduced phagocytic activity, linking IgG4 with a possible protective function in RTS,S vaccinees (30). IgG4 has the highest affinity to antigens compared to the other IgG subclasses, which could result in the ability to out-compete IgG1 and IgG3 even at low concentrations (24, 25) and may explain the different associations

with risk and protection. Thus, IgG4 would be protective by blocking opsonic phagocytosis mediated by cytophilic subclasses, which could be used by *Plasmodium* spp. as a means to shelter in phagocytes and then evade the immune system (30). In view of these results, we propose that this mechanism of IgG4 protection could apply to other non-vaccine related antigens and that protection needs a delicate balance combining the cytophilic activity elicited by IgG1 and IgG3 with the blocking activity of IgG4. This balance can be affected by factors such as the target antigen, age, MTI and RTS,S vaccination (56).

Unexpectedly, IgG1 and IgG3 levels to some antigens (DBLα, EBA140 R3-5, EBA175 R3-5, MSP1 bl2 [3D7, Well, RO33, and Mad20 strains], MSP5, MSP6, RH2 2030, RH4.2, and SSP2) increased after RTS,S vaccination. In contrast, levels of IgG1 to exposure antigens (AMA1 and $MSP1_{42}$) decreased following RTS,S immunization, suggesting that RTS,S vaccinees were less exposed to the parasite due to vaccine efficacy. Importantly, among the antigens to which antibodies were higher after RTS,S vaccination, MSP1 bl2 Well, MSP2 FL Dd2, MSP5, and MSP6 showed higher levels in controls than in malaria cases and were associated with protection in adjusted models for IgG1 and some for IgG3, while IgG1 to AMA1 and RH1, which decreased or did not change with RTS,S vaccination, were associated with malaria risk. Similarly, higher M0 to M3 change of IgG3 levels to MSP1 bl2 and MSP5, which were increased by RTS,S vaccine, was observed in controls than malaria cases. In adjusted models, IgG3 to MSP1 bl2, MSP5 (in addition to VAR2CSA DBL1-2, which was not affected by RTS,S vaccine) were also associated with protection at M3. In contrast to IgG1, stratified analysis including only RTS,S vaccinees showed that IgG3 to PE antigens LSA1 and SSP2 and MSP1 bl2, the latter two increased after RTS,S vaccination, correlated with protection. For IgG3, associations with malaria risk were only found for MSP2 FL CH150, which was not affected by RTS,S vaccination. In multi-marker analysis of M3 antibody levels, IgG1 levels to $MSP1_{42}$ and IgG1 and IgG3 levels to MSP2 contributed significantly and positively to the components associated with malaria, whereas IgG3 levels to numerous antigens including VAR2CSA DBL1-2, MSP1 bl2 3D7, MSP5 contributed to the components in an inverse relationship, consistent with the malaria protective associations in previous results. In addition, IgG3 to pTRAMP, CelTOS, RH4.9, SSP2, and RH2 were also important variables in the components suggesting a role in malaria protection. RTS,S vaccination also decreased the levels of IgG2 to EXP1, another exposure antigen, but it increased the levels of IgG2 to VAR2CSA DBL3-4 and IgG2 and IgG4 levels to MSP5. However, these responses were not associated with protection against malaria.

The observation that a vaccine that reduces exposure to the parasite is associated with an increase in IgG1 or IgG3 levels to certain antigens, and that this increase may be associated with malaria protection, could be related to the fact that RTS,S does not result in sterile immunity, particularly during the course of primary vaccination; instead, it is a partially-effective or "leaky" PE vaccine. To explain the differences in duration of vaccine efficacy between two cohorts of diverse MTI in the Mozambican phase 2b clinical trial (57), we hypothesized that partial protection afforded by RTS,S/AS0 may stimulate protective antibodies to certain malaria target antigens, through a reduction of merozoite release from the liver, leading to attenuated BS parasitaemia (58). Partially controlled infection would result in subpatent low antigen doses that could elicit enhanced IgG production to certain antigens, which would reflect in accelerated acquisition of BS protective immunity (59). While we could not observe this effect in a past study assessing IgG responses to a different set of antigens 6 months after vaccination (59, 60), IgG1 and IgG3 data obtained here would fit with this hypothesis. Alternatively, the effect on antibody levels could be due to antigen-specific immune stimulation provided by the adjuvant. It appears unlikely that RTS,S would be inducing antibodies to CSP that cross-react with other merozoite antigens.

In addition, IgG1 to LSA1 and SSP2 and IgG4 to CelTOS and a trend for LSA1, were associated with protection in RTS,S vaccinees in multivariable analyses. Multi-marker analysis also revealed a significant contribution of IgG3 to pTRAMP, CelTOS, and SSP2 (which were increased after RTS,S vaccination) to protection. We wondered how RTS,S might benefit the response to other PE antigens and a protective effect in RTS,S vaccinees. We speculate that the anti-CSP antibodies induced by the vaccine prevent the quick invasion of the hepatocytes (61), and this could allow a longer exposure of the sporozoites to the immune system, phagocytosis and antigen presentation to T cells, facilitating the response to PE antigens, which could have a synergistic effect with anti-CSP antibodies. When stratified by age and site at M3, an interesting pattern appeared for IgG4 to these and other antigens: children with malaria in the RTS,S group had lower levels of IgG4 while children with malaria who received the comparator vaccine had higher IgG4 levels. This suggests that there might be an RTS,S effect in the association of IgG4 responses with protection.

Age and site also affected IgG4 responses, children showing higher levels than infants, and Manhiça higher levels than Kintampo. In general, levels for IgG subclasses (less evident for IgG2) increased with age and IgG4 was not an exception. In fact, a previous study showed that IgG4 levels to EBA175 increased with age (13). As for site, a lower MTI could favor IgG4 rather than high MTI because it has been shown that anti-inflammatory cytokines, such as IL-10, increase with decreasing MTI (62). IL-10 is a key cytokine inducing IgG4, further relating this subclass with anti-inflammatory, and immune regulation effects (63). Nonetheless, we acknowledge that a bias by site cannot be discarded as the design of the study had this limitation. It included mostly malaria cases in Kintampo, and mostly no malaria controls in Manhiça, most of whom were children and the few cases were mostly infants.

Some antigens showed remarkable patterns in this study. Particularly, the higher IgG_{1-4} levels to MSP5 after RTS,S immunization and their association with protection (64, 65). IgG1 to MSP1 bl2 constructs (66, 67) also increased post-vaccination and were associated with protection. Both are merozoite surface proteins and have been associated with reduced malaria, but they also have distinct features. MSP5 is a highly conserved antigen, whose function is unknown and is not essential for growth *in vitro* (68) although it has never been reported to be absent in sequenced field strains. In

contrast, MSP1 bl2 is a highly polymorphic region of the N-terminal of this large protein which C-terminal end mediates the initial steps of merozoite invasion (68). MSP5 mainly induces IgG1 whereas MSP1 bl2 mainly elicits IgG3. An explanation for this is given by the conservation theory, which says that conserved antigens mainly elicit IgG1 whereas polymorphic antigens tend to elicit IgG3 (69) although this is not a consistent finding; for example, antibodies to the conserved regions of EBA175, EBA140, EBA181, are mainly IgG3 (70). Another explanation is that antibodies to structured regions or proteins are IgG1-skewed and antibodies to disordered proteins or regions are IgG3-skewed. Due to the peculiar pattern of naturally-acquired antibody responses to MSP5 and MSP1 bl2 upon RTS,S vaccination and their association with protection, their potential role in the efficacy and duration of RTS,S vaccine immunity and as adjunct or combination vaccines merit further study.

To conclude, we found that malaria protection was associated with cytophilic IgG3 levels mostly, IgG1 levels and, unexpectedly, with IgG4 responses to vaccine-unrelated antigens, specially MSP5 and MSP1 bl2, but also PE proteins, particularly in RTS,S vaccinees. These antigens are candidates to be included in multivalent and multistage second-generation vaccines that could attain higher efficacy than current products. In contrast, IgG2 responses were associated with higher risk of malaria. Our results highlight the need to assess subclass responses to malaria antigens in addition to total IgG responses since their levels and effector mechanisms are different, with subsequent impact on malaria protection. Importantly, the data of this study provides a better understanding of the interaction between NAI and RTS,S/AS01E-induced immunity. According to our results, NAI contributes to the protection afforded by RTS,S and RTS,S vaccination affects the acquisition of NAI against PE and BS antigens, increasing responses to those that are primarily associated with protection against malaria.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with ICH Good Clinical Practice guidelines and the Declaration of Helsinki. Parents/guardians of infants and children gave written informed consent for participation in the study. The protocol was approved by the following ethics committees or IRBs: Kintampo Health Research Centre (KHRC) Institutional Ethics Committee (IEC), Ghana; Noguchi Memorial Institute for Medical Research IRB, Ghana; Comité Ètic d'Investigació Clínica (CEIC, Hospital Clínic, UB), Spain; Comite Nacional de Bioetica (CNBS), Mozambique; Research Ethics Committee (REC), PATH, USA.

AUTHOR CONTRIBUTIONS

CD, RS, and GM wrote the first draft. CD, JC, and GM conceived the study. RS, AA, IU, and CV performed database management, statistical analysis, and experimental design. AN, CJ, JC, GM, CD, DD, KA, BGy, and SO-A collected samples and data and participated in the clinical trial. MV, AJ, and IU performed the experiments. NW, ND-P, and CD coordinated the study. IU, CD, JC, GM, RA, and CV participated in the design of the analysis. DL, VC, CC, SD, DG, EA, BGa, RC, DC, JB, and LR provided antigens. JB contributed to the write up of the manuscript. All reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00439/full#supplementary-material

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Cerebral Malaria in Mouse and Man

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Cerebral malaria (CM) is an acute encephalopathy caused by the malaria parasite *Plasmodium falciparum*, which develops in a small minority of infected patients and is responsible for the majority of deaths in African children. Despite decades of research on CM, the pathogenic mechanisms are still relatively poorly defined. Nevertheless, many studies in recent years, using a combination of animal models, *in vitro* cell culture work, and human patients, provide significant insight into the pathologic mechanisms leading to CM. In this review, we summarize recent findings from mouse models and human studies on the pathogenesis of CM, understanding of which may enable development of novel therapeutic approaches.

Keywords: cerebral malaria, *Plasmodium falciparum*, *Plasmodium berghei*, blood-brain barrier, T cells

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INTRODUCTION

Malaria is a life-threatening disease with an estimated 216 million cases of disease and ~445,000 deaths in 2016 (1). The majority of cases of malaria are among children under the age of five and pregnant women in sub-Saharan Africa. Human malaria is caused by five different species of *Plasmodium* parasites, of which *Plasmodium falciparum* and *Plasmodium vivax* are the most prevalent and *Plasmodium falciparum* is the most lethal. Human infections can also be caused by *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* (2). Female Anopheles mosquitoes transmit malaria by injecting sporozoites into the host while taking a blood meal (3). These parasites travel to the liver and invade hepatocytes and then multiply and develop into schizonts. After about a week, the hepatic schizonts burst and release thousands of merozoites that invade erythrocytes. Within the erythrocyte, the merozoites begin the asexual cycle, which takes approximately 24 h for *P. knowlesi*, 48 h for *P. falciparum*, *P. vivax*, and *P. ovale*, and 72 h for *P. malariae* (4). The erythrocytic schizont then ruptures and releases merozoites that can invade erythrocytes and repeat the erythrocytic cycle. Some of the merozoites also develop into gametocytes that can transmit malaria to mosquitoes (3). The liver-stage of malaria is asymptomatic, with disease symptoms all deriving from the cycling of parasites in the blood (5, 6). The clinical presentation of malaria differs depending on the age of the patient and whether they have had previous exposure (7). Fully immune individuals in malaria endemic areas will largely endure asymptomatic malaria. Most patients suffer from uncomplicated malaria with mild symptoms such as fever, headache, chills and vomiting. Some patients with *Plasmodium falciparum* malaria develop severe complications like severe anemia, respiratory complications and acidosis or cerebral malaria. In adults, multi-organ failure is also frequent (8–10).

CEREBRAL MALARIA

Cerebral malaria (CM) is a severe neurological complication of infection with *Plasmodium falciparum*. It causes a diffuse encephalopathy associated with coma and is responsible for most malaria-related deaths globally. CM is defined by the WHO as a clinical syndrome characterized by coma with the presence of asexual forms of *Plasmodium falciparum* in peripheral blood, and exclusion of other factors that could cause unconsciousness such as other infections or hypoglycemia (2). CM causes 15–20% mortality, despite effective antimalarial therapy and intensive care, and survivors may develop long-term neurological deficits (11–13). This severe form of the disease is most frequent in sub-Saharan Africa, where malaria transmission is intense. In this region, CM principally occurs in children under five and is rare in adults. However, in South East Asia, where malaria transmission is low, CM principally occurs in adults (2). The clinical manifestations of CM differ between children and adults (14–16), suggesting that different pathophysiological features are associated with human cerebral malaria (HCM) depending on age. In adults, CM is often accompanied by multi-organ complications, including central nervous system dysfunction, liver dysfunction, acute kidney failure, and respiratory failure. In contrast, in African children, CM usually manifests as coma, seizures, and severe anemia, but respiratory and renal failure are generally rare (14, 17, 18). Retinal abnormalities including retinal hemorrhages, papilledema, retinal whitening and retinal vessel color changes are common in children with CM. Components of retinopathy can be useful for distinguishing malarial from non-malarial coma (19–23).

PATHOGENESIS OF CM

The precise mechanisms involved in the pathogenesis of HCM are not fully understood. Most observations regarding HCM have relied on examination of post-mortem samples. Sequestration of infected red blood cells (iRBC) in the brain of *P. falciparum*-infected people is a hallmark of CM in adults (24–27) and children (23), and has been proposed as the main process responsible for HCM development (28). The iRBCs that contain mature parasites disappear from the peripheral circulation and specifically localize in microvessels of the brain and other organs. Adhesion of iRBCs to vascular endothelium is mediated by *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1), a specific cell-surface ligand expressed by iRBCs. PfEMP-1 is able to bind to many host ligands on endothelial cells, such as CD36, the intercellular adhesion molecule 1 (ICAM-1) and the endothelial protein C receptor (EPCR) (29–32). It has been suggested that accumulation of iRBCs in the cerebral capillaries causes mechanical obstruction of the vessels leading to a reduction in blood flow, hypoxia, coma, and death (24). Many studies have reported that there is a significant correlation between sequestration of iRBCs in cerebral vessels and coma in patients with CM (24–27, 33). However, sequestration of iRBCs in the brain capillaries might not be essential to cause CM, because some patients clinically diagnosed with HCM had little or no sequestration of iRBCs in their brain (23). Some of these latter patients were clearly a consequence of misdiagnosis (23), supporting the view that parasite sequestration is essential for HCM.

A RODENT MODEL OF EXPERIMENTAL CM

A well-characterized model of experimental cerebral malaria (ECM), which utilizes *P. berghei* ANKA (PbA) infection of various mouse strains including C57BL/6, has been widely employed to dissect the mechanisms involved in CM. This ECM model shares some similarities with *P. falciparum* HCM (34). Here, susceptible mice infected with PbA develop neurological signs such as paralysis, ataxia, convulsion, and/or coma and die within the first 2 weeks of infection (35). However, in murine CM, sequestration of iRBC in the brain vasculature is not a major histopathologic feature. While some studies have reported that PbA iRBC can accumulate in different organs, including the murine brain (36, 37), a correlation between iRBC sequestration and ECM was not demonstrated (36, 37). Nevertheless, one study showed that accumulation of iRBC in the brain of mice is crucial for the development of ECM (38). Other studies used luciferase-expressing PbA to report that iRBC sequestration in various organs, including the brain, was associated with the onset of ECM (39, 40). They observed a rapid increase in parasite biomass in various organs of infected mice at the time mice developed clinical signs. More recently, Strangward et al. showed accumulation of iRBCs in brain blood vessels was a specific feature of ECM caused by PbA and was not observed during uncomplicated *P. berghei* NK65 infection (41). This study suggested that a single iRBC is sufficient to occlude a brain capillary in PbA-infected mice, implicating this process as a contributing attribute in the pathogenesis of ECM, thus paralleling human CM.

THE ROLE OF IMMUNE CELLS

Immune system effector cells have been proposed to be involved in the pathogeneses of CM. In addition to iRBCs, some studies have reported host cells such as leukocytes and platelets within brain microvessels of patients with CM (42–45), though this observation is not universal (24, 27). Grau et al. showed that platelet accumulation occurs in the cerebral microvasculature of Malawian children with CM and that platelets are colocalized with malaria pigment and white cells in most patients with CM (42). They have also reported that platelet accumulation in brain microvessels were significantly higher in patients with CM than in those with either severe malarial anemia or nonmalarial encephalopathies. Another autopsy study reported intravascular accumulations of monocytes and platelets in the brain microvasculature of children with fatal CM (45). This study also showed that children with autopsy-confirmed CM had significantly more brain intravascular monocytes and platelets than did children with other causes of death. In contrast, studies examining the brains of adult patients from Thailand, observed little or no accumulation of leukocytes or platelets within brain

tissue (24, 27). Intravascular accumulation of immune cells has been extensively observed in the brains of mice with ECM (46–48). These immune cells are mainly composed of T cells, neutrophils, monocytes, and natural killer (NK) cells. It has been shown that the migration of leukocytes to the brain occurs at the same time as the neurological signs of ECM appear. NK cells have been reported to be required for the development of ECM and are recruited to the brain of PbA-infected C57BL/6 mice (47). Here, depletion of NK cells using anti-asialo-GM1 antibody protected mice from CM by inhibiting the migration of $CD8^+$ and $CD4^+$ T cells to the brain. This result should be taken with some caution, however, since activated $CD8^+$ T cells can also express this marker and are essential for ECM.

The involvement of monocytes, macrophages and neutrophils in the pathogenesis of ECM is still unclear. Antibody depletion of neutrophils and macrophages shortly before manifestation of neurological signs did not prevent the development of CM in PbA-infected mice, suggesting that these cells are not involved in the effector phase of ECM (46). Pai et al. have shown that depletion of monocytes prior to PbA infection resulted in complete protection from ECM (49). However, depletion of monocytes late after PbA infection had no effect, indicating that monocytes are not essential to ECM pathology. Nevertheless, these mice showed a significant reduction in the number of $CD8^+$ T cells, $CD4^+$ T cells, and NK cells within the brain, suggesting that monocytes/macrophages play a limited role in the recruitment of lymphocytes to the brain during ECM. This limited role was further emphasized in a recent study that depleted neutrophils with anti-Ly6G antibodies in $CCR2^{-/-}$ mice (which also lack circulating monocytes) prior to PbA infection and showed this did not prevent ECM development (50).

Platelets have been implicated in ECM based on evidence of accumulation within brain microvessels of diseased mice (51). Intravital imaging of the brain of mice with ECM revealed small clusters of platelets were marginalized in post-capillary venules and that these clusters were co-localized with patches of P-selectin (52, 53). These observations may be important, as depletion of platelets by anti-CD41 mAb in early but not late stages of ECM development protected mice from disease, implicating platelets in ECM pathology (54, 55). Activation of platelets leads to the release of large amounts of CD40 ligand (CD40L, CD154), which may interact with CD40 constitutively expressed on endothelial cells (56, 57). Mortality and breakdown of the blood-brain barrier (BBB) were prevented in PbA-infected mice lacking either CD40 or CD40L (58). Macrophage sequestration was also reduced in brain vessels of these mice. While these data may implicate platelets and platelet-derived CD40L in ECM, caution should be taken in this interpretation as CD40 has other important roles in ECM, such as in the provision of $CD4^+$ T cell help for the generation of parasite-specific $CD8^+$ T cell responses (59). Supporting a role for platelet-derived CD40L, it has been shown that adoptive transfer of WT $CD40^+$ platelets into CD40-deficient mice, increased ECM mortality (60).

Accumulation of both $CD8^+$ T cells and $CD4^+$ T cells in the brain of PbA-infected mice has been described in several studies (46–48, 61, 62). A number of these and other reports have also demonstrated that $CD8^+$ T cells are required for the development of ECM. Antibody depletion of $CD8^+$ T cells in PbA-infected F_1 (129/Ola × C57BL/6J) and 129P2Sv/ev mice shortly before manifestation of neurological signs prevented the development of ECM (46, 48). Depletion of $CD8^+$ T cells in PbA-infected B6 mice 4 days after infection prevented the vascular hemorrhaging, BBB breakdown, and the development of ECM (50). CD8-deficient (40, 46, 63) or β2-microglobulin-deficient mice, which lack functional $CD8^+$ T cells (64) also failed to develop ECM. These results suggest that brain-sequestered $CD8^+$ T cells play an important effector role in the development of ECM. This was confirmed by adoptive transfer experiments where splenic $CD8^+$ T cells or $CD8^-$ T cells from PbA-infected C57BL6 mice were transferred into ECM-resistant, RAG2-KO recipient mice that were subsequently infected with PbA (61). RAG2-KO recipient mice developed ECM after transfer of $CD8^+$ T cells, but not after transfer of $CD4^+$ T cells, suggesting that cytotoxic $CD8^+$ T cells are critical for the development of ECM. In addition, adoptive transfer of $CD8^+$ T cells isolated from perforin-deficient mice into RAG2-KO recipient mice did not cause ECM (61). These results suggest that perforin-dependent cytotoxic pathways are involved in the pathogenesis of ECM. It has also been shown that expression of granzyme B by $CD8^+$ T cells is essential for the development of ECM (65). Granzyme B-deficient mice had significantly lower total parasite burdens in the brain and were completely resistant to ECM. However, similar numbers of infiltrating $CD8^+$ T cells were found in the brains of PbA-infected wild-type and granzyme B-deficient mice (65), suggesting granzyme B was required in the effector phase rather than in development or sequestration of $CD8^+$ T cells to the brain. $CD8^+$ T cells have been shown to promote iRBC accumulation in the brain and other organs during ECM (39, 40). It has been shown that significantly fewer iRBC accumulated in brains, spleens and other organs of $CD8^+$ T cell-depleted mice than in WT or $CD4^+$ T cell-depleted mice (40). Baptista et al. have shown that depletion of $CD8^+$ T cells a day prior to the onset of ECM protected PbA-infected mice and reduced the accumulation of iRBC in the brains of those mice (38). They have also shown that treatment with antimalarial drugs delayed development of ECM in PbA-infected mice without altering the number of $CD8^+$ T cells in the brain (38). These results suggest that the brain sequestration of $CD8^+$ T cells is not sufficient for the development of ECM and that the presence of both $CD8^+$ T cells and iRBC in the brain is crucial. Baptista et al. (38), also reported the accumulation of $CD8^+$ T cells in the brains of mice infected with *P.berghei* NK65 (a parasite line that does not cause ECM). However, neither accumulation of iRBCs in the brain nor BBB breakdown was observed in these mice, suggesting iRBC accumulation is important for the onset of ECM. The requirement of iRBC accumulation may underpin a need for presentation of parasite antigens by MHC I molecules on brain endothelium (50). Parasite-specific T cells were shown to spend greater time than non-specific T cells arrested on brain blood vessels of mice undergoing ECM and this interaction could be impaired by antibody that blocked recognition of MHC I-peptide complexes. Abrogation of ECM in chimeric mice where

brain endothelium lacked expression of MHC I molecules further supports this view.

It has been shown that $CD4^+$ T cells can either play a role in the induction phase or in both the induction and in the effector phase of ECM. Antibody depletion of $CD4^+$ T cells in PbA-infected F_1 (129/Ola X C57BL/6J) (46), CBA/Ca (66), and C57BL/6 mice (39, 64) prevented the development of ECM when conducted before or early in the infection, suggesting an essential role for $CD4^+$ T cells in the initiation of ECM pathogenesis. A role for $CD4^+$ T cells in ECM pathogenesis was also supported by experiments where PbA infection of CD4-deficient mice did not lead to the development of ECM (46, 63, 64). A recent study has reported that depletion of $CD4^+$ T cells 4 days after infection did not protect PbA-infected B6 mice from ECM (50). Given the importance of $CD8^+$ T cells in ECM pathogenesis and the well-established role for $CD4^+$ T cells in helping $CD8^+$ T cells in many infection models (67), the requirement for $CD4^+$ T cells in ECM pathogenesis may reflect a similar helper role. This view is strongly supported by the capacity of wild-type PbA-specific $CD4^+$ T cells to provide help for $CD8^+$ T cell expansion and ECM development in CD40 ligand-deficient mice, where endogenous $CD4^+$ T cells lack expression of this receptor (59). The role of $CD4^+$ T cells in the effector phase of ECM is still unclear. In some studies, using C57BL/6 or (129/Ola x C57BL/6) F_1 mice, depletion of $CD4^+$ T cells immediately before the onset of neurological signs did not prevent development of ECM (40, 46). However, for 129P2Sv/ev mice, depletion of $CD4^+$ T cells late after PbA infection prevented the development of ECM (48). In another study, the majority of C57BL/6 and C57B1/10 mice infected with PbA were protected from ECM development when $CD4^+$ T cell depletion was undertaken just prior to normal disease onset (68). Thus, while all studies agree with a requirement for $CD4^+$ T cells, whether this is largely during the priming phase or in part contributed in the effector phase may depend on the strain of mice and perhaps the environment where experiments are performed.

THE ROLE OF CYTOKINES, CHEMOKINES, AND ADHESION MOLECULES

Pro-inflammatory cytokines such as lymphotoxin α (69) and IFN-γ (70, 71) have been implicated in the development of ECM during PbA infection. Elevated blood concentrations of cytokines, particularly TNF and IFN-γ were found in patients with CM (72–76) and also in animal models of ECM (71, 77). An essential role for TNF has been excluded for ECM, however, since TNF-deficient mice are susceptible to PbA-induced disease (69). The key role of IFN-γ in the pathogenesis of ECM was confirmed by experiments where PbA-infected IFN-$\gamma^{-/-}$ and IFN-γ receptor (IFN-γR)$^{-/-}$ mice did not develop ECM, nor show parasite or leukocyte accumulation in their brains (48, 71). IFN-γ was shown to be involved in the control of iRBC accumulation in the brain and other organs of PbA-infected mice (39, 40). IFN-γ also promotes the up-regulation of adhesion molecules such as ICAM-1 on brain endothelial cells during malaria infection (71, 78). Cerebral endothelial cells from PbA-infected IFN-$\gamma^{-/-}$ mice showed significantly reduced expression of adhesion and antigen presenting molecules when compared to wild type mice (50). It has been shown that IFN-γ can be produced in response to *Plasmodium* parasites by many cell types, including NKT cells, NK cells, γδ TCR^+ T cells, and αβ TCR^+ $CD4^+$ and $CD8^+$ T cells (79). Adoptive transfer of infection-derived $CD4^+$ T cells, but not innate or $CD8^+$ T cells, into normally resistant IFN-$\gamma^{-/-}$ mice (infected with PbA) promoted the development of ECM by active secretion of IFN-γ, implicating cytokine derived from $CD4^+$ T cells in ECM (80). This conclusion was further supported by showing that IFN-γ-producing $CD4^+$ T cells enhanced the expression of CXCL9 (Mig) and CXCL10 (IP-10) and induced $CD8^+$ T cell migration and accumulation within the brain of PbA-infected IFN-$\gamma^{-/-}$ mice.

Endothelial cell adhesion molecules are believed to play an important role in the pathogenesis of cerebral malaria. Elevated circulating levels of pro-inflammatory cytokines including IFN-γ and TNF during malaria infection result in an intense up-regulation of endothelial cell adhesion molecules such as ICAM-1, VCAM-1, and P-selectin in PbA-infected mice (78). Marked up-regulation of ICAM-1, VCAM-1, and E-selectin on brain endothelial cells has also been observed in patients with CM (81–83). Postmortem studies have shown that there is co-localization between sequestered iRBCs and ICAM-1 in the cerebral vessels (82). The importance of ICAM-I in the development of ECM was confirmed by experiments where PbA-infected ICAM-$1^{-/-}$ mice did not develop ECM (84). This was associated with a slight reduction in macrophage sequestration to the brain and an absence of BBB breakdown (84). In another study (50), late treatment of PbA-infected mice with a combination of anti-LFA-1 and anti-VLA-4 antibodies was shown to disrupt the $CD8^+$ T cell interactions with brain endothelial cell expressed ICAM-1 and VCAM-1, resulting in a rapid displacement of PbA-specific $CD8^+$ T cells from the cerebral vessels, protecting mice from ECM.

CXCR3, a T cell chemokine receptor, has been shown to be necessary for recruitment of T cells into the brain and the development of ECM (85). A majority of PbA-infected CXCR3-deficient mice did not develop ECM and showed reduced numbers of $CD8^+$ T cells in their brain vessels (54, 62, 85). Enhanced expression of CXCR3-binding chemokines such as CXCL9 and CXCL10 has been reported in the brains of PbA-infected mice and mice deficient in either chemokine were partially protected from ECM (85). Mice lacking CXCL10 were also shown to have increased retention of T cells in the spleen and reduced T cell infiltration in the brain, coinciding with reduced disease (86). A recent study has also reported that CXCL10 produced by brain endothelial cells induces the adhesion of T cells to cerebrovascular endothelium and prevents T cell detachment from the brain vasculature in PbA-infected mice. The induction of CXCL10 was shown to be IFN-γ dependent (87).

CXCL4, which is also a ligand for the chemokine receptor CXCR3, is released by activated platelets early in the course of ECM (54). CXCL4 has been shown to be necessary for the

development of ECM, as the majority of mice deficient in CXCL4 are protected from disease (54). It was shown that CXCL4 stimulates the production of TNF by T cells and macrophages as well as inducing T cell migration to the brain.

THE BLOOD-BRAIN BARRIER (BBB) IN CM

The BBB is an interface between the intravascular space and the central nervous system (CNS) that regulates passage of molecules from the blood into the brain and the transport of carbon dioxide and metabolic waste products from the brain into the blood (88). There are two functionally distinct blood brain barriers (89); (i) a physiological BBB that regulates diffusion of solutes and is formed by capillaries - it encompasses a single layer composed of endothelial cells, gliovascular membrane, and astrocyte endfeet of the glia limitans, and (ii) a neuroimmunological BBB, which is formed by post-capillary venules and consists of two layers, the vascular endothelium with its basement membrane and the glia limitans with associated astrocyte endfeet and their basement membrane. These two layers are separated by perivascular space. In an inflammatory response, immune cells migrate into the CNS at the neuroimmunological BBB. Here, immune cells need to cross two physical barriers, the vascular endothelium and the glia limitans to enter the CNS parenchyma (89, 90). Whether lymphocyte crossing of the BBB is essential for development of ECM is unclear. Evidence that blocking the adhesion molecules LFA-1 and VLA-4 to release $CD8^+$ T cells accumulating within the lumen of blood vessels prevents ECM, yet has no effect on $CD8^+$ T cells within the brain parenchyma (50), suggests those cells that cross the BBB are not essential for disease. What appears to be more important is breakdown of solute exclusion by the BBB and consequent swelling of the brain.

Many studies have examined BBB alterations in *P. falciparum* patients by measuring the level of molecules such as albumin or immunoglobulins (IgG) in the cerebrospinal fluid (CSF) and plasma. Albumin is not synthesized intrathecally and is excluded from the brain by an intact BBB whereas IgG can be synthesized intrathecally by plasma cells. Calculation of the albumin index, $[\text{albumin}]_{CSF}/[\text{albumin}]_{plasma}$, is used to examine BBB integrity in individuals. Calculation of the IgG index, $([\text{IgG}]_{CSF} \times [\text{albumin}]_{plasma}/[\text{IgG}]_{plasma} \times (\text{albumin})_{CSF})$ is a traditional method of detecting intrathecal IgG production (91). It has been shown that radioactive ^{125}I-labeled albumin levels in the CSF of Thai adult patients with CM were not increased after injection during and after coma (92). In another study, albumin and IgG levels in the CSF of Vietnamese adult patients with CM were not elevated compared with control subjects, except in a few individual cases (93). In contrast, Malawian children with CM showed higher levels of albumin in the CSF compared with UK adult controls, although no difference was found in CSF albumin levels between children who died vs. those who survived from the disease (94). Measuring the local synthesis of IgG within the CNS showed an increased IgG index in the CSF of 43% of adult Thai patients with CM (95). All together, these studies suggest that some degree of permeabilisation of the BBB occurs in individual patients with CM.

Postmortem studies of the brains of Malawian children who died from *P. falciparum* malaria have shown the presence of myelin and axonal damage, BBB breakdown, and glial responses, in addition to the sequestration of iRBCs in brain microvessels (44). Axonal damage, demyelination, and ring hemorrhages have also been observed in the postmortem brain tissue of Vietnamese adult patients who died from *P. falciparum* malaria (96). Another recent study directly compared retinal and cerebral histopathological changes in the same patients who died from CM. They found similar pathological features including hemorrhages, sequestration of iRBCs, axonal damage, and BBB disruption in the retina and the brain of individual patients with fatal CM (97). The alteration of the BBB in mice with ECM was confirmed by measuring the movement of the dye Evans Blue, radio-labeled albumin, radio-labeled antibody or horseradish peroxidase (52, 98, 99). Several pathologic changes including brain edema, enlarged perivascular spaces, BBB breakdown, and vascular leakage have been observed in mice with ECM (52, 100, 101). ECM studies have also revealed evidence of cell death as well as vascular leakage for multiple brain regions, particularly in the brainstem and olfactory bulb (50). In this study, the majority of the dead cells in the brainstem were shown to be neurons. Cell death within the brainstem of mice undergoing ECM is likely caused by brain swelling, which parallels observations in CM patients, where brain swelling caused by edema leads to lethal cerebral herniation causing damage to this region (102).

The exact mechanisms responsible for the BBB alterations are not fully characterized. A reduction in expression of the endothelial tight junction–associated proteins, which are important for maintaining BBB integrity, has been observed in vessels of human brains, where they colocalize with areas of sequestered iRBCs (94, 103). A reduction in expression of endothelial tight junction proteins in areas of vascular breakdown in the brain has also been observed for mice undergoing ECM (50). These data suggest modulation of tight-junction-associated protein expression may contribute to BBB alterations, possibly by disrupting the connections that maintain BBB integrity.

In vitro studies have shown that the capture of iRBC-associated material by brain endothelial cells leads to the opening of intercellular tight junctions (104). Capture of parasite material *in vivo* has also been shown to enable brain endothelial cells to cross-present PbA antigens on MHC I, in an IFN-γ-dependent manner (105). Recognition of these antigens by $CD8^+$ T cells *in vivo* also affects tight junction integrity (50). Monocyte adherence to endothelial cells in the retinal vessels is also accompanied by an increase in BBB permeability (106, 107), potentially implicating these cells in this process. Platelet adhesion to brain endothelial cells also seems to contribute to alteration of the BBB. It has been shown that platelets can act as a bridge between iRBC and the surface of brain endothelial cells and may therefore promote the adhesion of iRBC to the endothelial vascular (108). Low plasma platelet counts and binding of iRBC to EPCR by PfEMP1 has been linked to brain swelling and retinopathy in CM patients, suggesting that binding of platelets may precipitate or contribute to endothelial disruption and edema (109). Platelets were also found to potentiate apoptosis of TNF-stimulated

human brain microvascular endothelial cells through TGF-β *in vitro* (110). EPCR binding by iRBC has also been implicated in BBB breakdown through a local reduction in the generation of activated protein C causing down-stream effects on protease activated receptor 1 (PAR1) that alter vascular permeability (111).

Matrix metalloproteinases are zinc-dependent endopeptidases that are involved in many aspects of immunity (112). These enzymes can degrade extracellular matrix proteins as well as non-matrix targets, such as secreted cytokines, chemokines, and cell surface receptors (113). Elevated MMP expression or activity has been implicated in many disease processes (114). It has been shown that MMP-9 is crucial for the disruption of the BBB in several CNS diseases (115, 116). MMP-2 and MMP-9 activity at the parenchymal border are crucial for infiltration of leukocytes into the brain parenchyma in a mouse experimental autoimmune encephalomyelitis (EAE) model (117). MMP-2 and MMP-9 double knockout mice are resistant to EAE and leukocyte infiltration into the CNS is prevented in these mice (117). Elevated levels of several MMPs have been reported during ECM in different organs and inhibition of such MMPs with BB-94, a broad-spectrum MMP-inhibitor, delayed the onset of ECM by 1 day (118). There was a significant increase in the expression of activated forms of MMP-9 in the brain of PbA-infected C57/BL6 mice late in disease. However, no significant differences in lethality were observed between MMP-9 knockout mice and wild type mice during the course of ECM, suggesting that this MMP does not play a key role in the pathogenesis of ECM.

IMAGING APPROACHES FOR INVESTIGATING THE PATHOGENESIS OF CM

Several imaging techniques have been used to investigate the pathogenesis of cerebral malaria in living subjects. Computed tomography (CT) scans and magnetic resonance imaging (MRI) have provided some insights into human CM pathogenesis. Acute head CTs revealed some of the pathological changes in the brains of children with retinopathy-confirmed cerebral malaria including large vessel infarcts, edema, and herniation. Follow-up CT scans in survivors identified focal cortical atrophy that correlated with regions affected by focal seizures during acute cerebral malaria (119). A large MRI study was performed in Malawian children with CM and compared findings in unconscious patients with and without retinopathy. MRI findings revealed a wide range of abnormalities including brain swelling and severe edema, which were more common in patients with malaria retinopathy (120). In another MRI study, African children who met the definition of cerebral malaria and had retinopathy underwent MRI examination (102). MRI results showed that the majority (84%) of children who died from CM had severe brain swelling at admission. In contrast, evidence of severe brain swelling was only observed in 27% of survivors, and in these cases temporal MRI imaging showed that swelling was transient.

In a recent study, MRI was performed in adult Bangladeshi patients with severe *falciparum* malaria (121). Diffuse mild brain swelling, mostly without edema, was a common abnormality observed in patients with severe *falciparum* malaria and was not specific to patients with coma or fatal disease. The majority of patients had malarial retinopathy. Retinal changes were more severe and common in patients with coma (121). While these approaches provide valuable information regarding the pathological changes in the brains of malaria patients, they are limited in their capacity to investigate pathogenic mechanisms at a cellular and molecular level.

In mice, ultra-high-field MRI was used to identify the olfactory bulb as a vulnerable part of the brain during PbA infection (122). MRI images showed that micro-hemorrhages occur in the olfactory bulb when ECM symptoms begin, prior to other parts of the brain showing clear evidence of bleeding. Whole animal imaging is a technique that enables the visualization of parasite load in different organs of intact malaria-infected animals by using luciferase expressing parasites. While this approach is not suitable for monitoring individual parasites or immune cells, it has been of value to show a rapid increase in parasite biomass in the brains of mice at ECM onset (38–40).

Intravital microscopy (IVM) enables the visualization of individual cell interactions in live animals and is a useful tool for investigating the pathogenesis of ECM. A recent IVM study has suggested that ECM correlates with widespread opening of the neuroimmunological BBB and that this occurs without widespread loss of vascular endothelial cells in the brain (52). This study shows that mice with ECM, but not hyperparasitemia, exhibit leukocyte arrest, CD14 expression, platelet marginalization, and vascular leakage from post-capillary venules, but not capillaries or arterioles. Another IVM study from the same group reported the accumulation of numerous $CD8^+$ T cells, neutrophils, and macrophages within post-capillary venules of mice with ECM (53). Others showed that monocytes start to accumulate in the brain blood vessels 1–2 days prior to the onset of ECM and that activated $CD8^+$ T cells regulate monocyte accumulation in mice during ECM (49). As the disease progressed in PbA-infected mice, a significant reduction was observed in rolling velocity of monocytes, which was accompanied by a significant increase in the number of monocytes adhering to the microvasculature of the brain (49). Examination of the location of $CD8^+$ T cells within the brain revealed that the majority of infiltrating T cells accumulate on the perivascular side of the blood vessels, but this was only seen in mice infected with PbA but not Pb NK65 parasites (123). The accumulating $CD8^+$ T cells within the brains of mice infected with both Pb ANKA and Pb NK65 were similarly activated, but they exhibited different movement characteristics. This study also showed that infected red blood cells accumulated both in intravascular and perivascular spaces at the time of ECM development in PbA-infected mice. Significantly less parasite accumulation was observed in the brains of mice infected with Pb NK65 potentially explaining the lack of ECM in this model.

Swanson et al. reported that PbA-specific $CD8^+$ T cells arrested along both the luminal and extravascular surfaces of cerebral vasculature at the peak of ECM, although the majority interacted on the luminal side (50). They found that the arrest of PbA-specific $CD8^+$ T cells was specific for

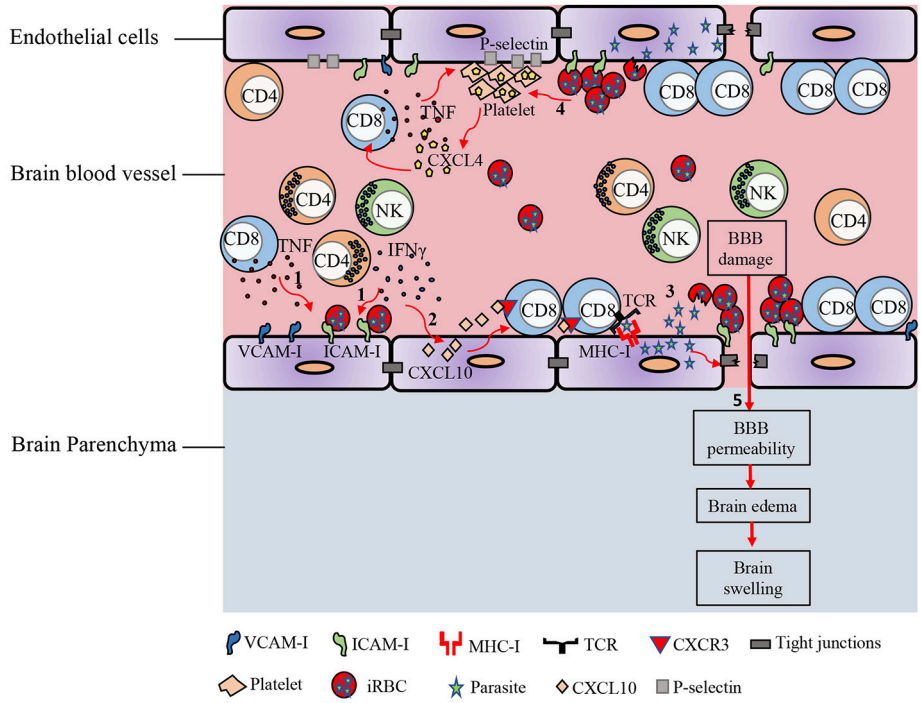

FIGURE 1 | Immunopathology of ECM. In response to *Plasmodium* blood-stage infection, pro-inflammatory cytokines such as IFN-γ and TNF are produced by various immune cells. **(1)** These cytokines promote the up-regulation of adhesion molecules on brain endothelial cells. iRBCs sequester in the cerebral microvasculature and bind to receptors on the endothelial cells such as ICAM-1. **(2)** IFN-γ induces the expression of CXCL10 by brain endothelial cells. IFN-γ-induced CXCL10 enhances the adhesion of T cells to cerebrovascular endothelium and prevents their detachment from the brain vasculature. **(3)** Parasite antigens can be transferred from the iRBC into the endothelial cell. This interaction leads to opening of the intercellular tight junctions. Brain endothelial cells can phagocytize parasite antigens and cross-present them to $CD8^+$ T lymphocytes. **(4)** iRBC can also directly activate platelets and stimulate the release of CXCL4 early in the course of ECM. CXCL4 induces the production of TNF by T cells and macrophages, which then causes more platelets to adhere to the endothelium. Enhanced expression of CXCR3-binding chemokines such as CXCL9, CXCL10, and CXCL4 in the brains of PbA-infected mice induces T cell migration to the brain in the late stage of ECM. **(5)** Accumulation of iRBCs and immune cells in cerebral vessels leads to vascular obstruction, increased pressure within the brain blood vessels, and increased permeability of the BBB, facilitating edema, and brain swelling.

brain vasculature and was not observed in other peripheral tissues. iRBCs were also observed on both the luminal and abluminal surfaces of cerebral blood vessels of these mice and were actively phagocytosed by myelomonocytic cells and perivascular macrophages. Importantly, interactions of parasite-specific $CD8^+$ T cells with the luminal side of brain blood vessels was impaired by blocking interactions with MHC I molecules, which together with the lack of ECM development in chimeric mice where MHC I was not present on endothelial cells or where T cell adhesion was inhibited by blocking the integrins VLA-4 and LFA-1, strongly implicated cognate interactions of $CD8^+$ T cells with brain endothelium in the process of ECM development. Together, intravital imaging studies highlight the contribution of sequestered iRBCs and brain-infiltrating immune cells to the pathogenesis of ECM.

SUMMARY

Mouse models of ECM have been heavily criticized in the past for their lack of relevance to human CM, but recent studies (41, 50, 52, 53), suggest CM and ECM may be more similar than previously appreciated. Accumulation of iRBC and platelets in the brain, breakdown of the BBB, and swelling of the brain and its subsequent lethal consequences, all appear to be common attributes of both species. Recent studies in mice suggest techniques available to assess $CD8^+$ T cell accumulation in human brains are inefficient and struggle to detect the relatively small number of infiltrating $CD8^+$ T cells in histological sections (41), thus leaving this component of murine pathology potentially still relevant to human disease. A current simplified model of the pathogenesis of cerebral malaria suggests several important factors interplay to cause lethality (**Figure 1**). The primary requirement is sequestration of parasites in the brain. This is mediated by direct binding of iRBC to brain endothelium or through such interactions mediated by accumulating platelets. These iRBC-endothelial cell interaction leads to breakdown of the control of solute movement across the BBB, particularly within the post-capillary venules. This process, at least in mice, is amplified by $CD8^+$ T cell recognition of parasite antigen on endothelial cells, possibly affecting the endothelial cells themselves or simply increasing cellular accumulation within the vessels. The accumulation

of these and other leukocytes as well as iRBC within post-capillary venules increases blood pressure within the brain, further driving edema and ultimately leading to sufficient brain swelling to cause lethal herniation. While some direct killing of endothelial cells may be mediated by $CD8^+$ T cells, causing overt bleeding, the primary mode of damage relates to increased permeability of the BBB, and increased pressure within the brain blood vessels, facilitating edema. Deciphering the precise mechanisms underlying the pathogenesis of CM is required for developing therapeutic approaches that can act to reduce or prevent death. Utilizing several different investigative approaches has improved our understanding of the cellular and molecular mechanisms underlying this disease. Use of new imaging methods and elucidation of the mechanistic basis of ECM in murine models may help better understand the spectrum of human disease and facilitate approaches for prevention of CM in humans.

AUTHOR CONTRIBUTIONS

NG wrote the paper. SM and WH contributed to writing and critically revised the paper.

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REVIEW
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The Ins and Outs of Cerebral Malaria Pathogenesis: Immunopathology, Extracellular Vesicles, Immunometabolism, and Trained Immunity

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Complications from malaria parasite infections still cost the lives of close to half a million people every year. The most severe is cerebral malaria (CM). Employing murine models of CM, autopsy results, *in vitro* experiments, neuroimaging and microscopic techniques, decades of research activity have investigated the development of CM immunopathology in the hope of identifying steps that could be therapeutically targeted. Yet important questions remain. This review summarizes recent findings, primarily mechanistic insights on the essential cellular and molecular players involved gained within the murine experimental cerebral malaria model. It also highlights recent developments in (a) cell-cell communication events mediated through extracellular vesicles (EVs), (b) mounting evidence for innate immune memory, leading to "trained" increased or tolerised responses, and (c) modulation of immune cell function through metabolism, that could shed light on why some patients develop this life-threatening condition whilst many do not.

Keywords: cerebral malaria (CM), immunopathology, extracellular vesicle (EV), Immunometabolism, trained immunity

INTRODUCTION

Among protozoan parasites of the genus *Plasmodium*, seven species are able to infect humans to cause malaria. The two major species are *Plasmodium vivax* and *Plasmodium falciparum*, with the former accounting for the most cases and the latter being responsible for the majority of deaths (1). Plasmodia are dual host parasites (mosquito and mammals) and within the mammalian host, broadly two hepatic stages and one blood stage are defined. Malaria induces a wide spectrum of symptoms and signs which differ between affected individuals, for example, between adults and children. In 1–2% of cases however (2), infection leads to severe malaria that exclusively develops during the blood stage of the malaria parasite cycle. Severe malaria can include the following disturbances, singly or in combination: electrolyte and metabolite imbalance, severe anemia, pulmonary oedema with respiratory distress, jaundice, and its most severe manifestation: cerebral malaria (CM). CM is characterized by, seizures, coma, and death.

In endemic regions of Africa, CM mostly affects children under the age of five while in Southeast Asia, it is observed mostly in young adults. CM lethality still is in the range of 15–25% with the best available treatments (3). Over 25% of survivors from CM are afflicted by life-long sequelae including sensory and cognitive impairment (4), epilepsy and physical disability. Thus, despite incremental progress in more than a century since the discovery of the malaria parasite, a deep understanding of this disease and its deadliest complication, CM, is far from complete, and development of effective therapies remains a high priority.

A comprehensive appraisal of the histopathology associated with CM or experimental animal models of CM, as well as its changes following characterized interventions, constitute a valid experimental approach to identify targetable disease development stages, cellular or molecular players. This could eventually lead to the identification of adjunctive therapies. Investigating the histopathology and pathophysiology of HCM (human cerebral malaria) in patients has ethical and technical constraints since pathological analysis of brain and most other tissues besides blood are limited to post-mortem observations. Thus, models of experimental cerebral malaria (ECM), caused by infection of susceptible mice with *Plasmodium berghei* ANKA (PbA), have provided important clues. ECM and HCM have more than twenty documented shared pathological features (5, 6). In both humans and mice, early lesions involve binding of infected erythrocytes (IE) to the brain microcirculation (7), albeit this is a minor feature in ECM compared to HCM. More recently the presence of IE in ECM has been confirmed (8), and found to correspond with development of vascular leakage. A cascade of sequestration of various leukocyte subtypes, including monocytes, macrophages, NK cells, $CD4^+$ and $CD8^+$ T cells, then ensues. In ECM, $CD8^+$ T cells are eventually responsible for endothelial damage and the subsequent breakdown of the blood-brain barrier (BBB). In both patients and mice, neurological signs progress from seizures, ataxia and paralysis to coma and eventual death, and both are associated with brain oedema and hemorrhages.

In this review, we summarize the consensus and controversies surrounding the sequence of cellular events leading to the development of vascular neuropathology in experimental CM (ECM) and their correlates in human CM (HCM). Numerous aspects of the similarities and differences between HCM and ECM have been reviewed (5, 6, 9–13), clearly stating the limitations as well as the strengths of mouse model. A consensus paper about these issues has been published (14). Other recent findings in HCM have been detailed and discussed (15). We also highlight recent investigations extending beyond the targeting of specific cell types (e.g., monocyte subsets) into potential modulators or effectors of CM such as extracellular vesicles, diet, immunometabolism and trained immunity.

SEQUENCE OF EVENTS LEADING TO CM IMMUNOPATHOLOGY

Both HCM and ECM are associated with a sequential and marked accumulation of various immune cell subtypes in the brain microcirculation but histological studies paint somewhat different pictures between them. In ECM, a number of studies have provided evidence for an early requirement for $CD4^+$ and for a late accumulation of $CD8^+$ T cells while for other accumulating cell types, there are still controversies as to direct pathogenic roles (5, 6, 16, 17). HCM is characterized by a disease spectrum in children that differs from what is observed in adults. Three disease categories have been defined in pediatric HCM (CM1, CM2, and CM3) (18), occurring in 15, 56, and 29% of clinically defined cases, respectively. CM1 patients only display IE sequestration, whereas patients with CM2 have IE sequestration associated with other intra- and perivascular pathology, including immune cell infiltrates. Patients with CM3 fulfill the complete World Health Organization clinical criteria for CM, including unarousable coma associated with infection, but died of non-malarial causes. In South-East Asian adult patients, mainly IE sequestration has been observed (19). Here we summarize the elucidated sequence of these cellular events and the proposed roles attributed to different cell types.

ACTIVATION OF ENDOTHELIAL CELLS AND SEQUESTRATION OF INFECTED ERYTHROCYTES AND PLATELETS

A hallmark in HCM post-mortem observations is the distension of cerebral capillaries and venules containing IE and platelets. This sequestration involves predominantly late stage IE and is considered an immune evasion mechanism, as it removes mature stages of the parasites form the circulation. It is accompanied by systemic endothelial activation with upregulation of markers of endothelial cell (EC) activation such as ICAM-1, E-Selectin, CD36, and von Willebrand Factor (vWF), that all can serve as adhesion molecules for IE. Platelets are now considered key contributors to CM by providing an alternate, indirect mechanism for IE cytoadhesion (20). This is achieved by forming bridges between IE and ECs at sites of low endothelial adhesion molecule expression, as shown *in vitro* (21, 22). These *in vitro* results have been confirmed by the demonstration of intravascular platelet accumulation in HCM, as shown in Malawian (3, 23) and South-East Asian (19) patients. Aside from this pathogenic role, platelets can exert a protective effect in malaria, as shown *in vitro* (24) and *in vivo* (25, 26). More recently, these *in vivo* results have been challenged, both *in vitro* using *P. falciparum* IE and *in vivo* using *P. chabaudi* and *P. berghei*-infected mice. Results indicate platelets do not kill blood-stage Plasmodium at physiologically relevant effector-to-target ratios. Furthermore, adoptive transfer of wild-type platelets to CD40-KO mice, which are resistant to ECM, partially restored ECM mortality and signs in CD40-KO recipients, indicating platelets are integral to the pathogenic process, and platelet CD40 is a key molecule (27), confirming 2002 data of Piguet et al. (28), *vide infra*. Sequestration of IE and platelets during ECM was originally disputed but further observations have also documented these as key pathogenic elements, as are the associated activation of ECs and upregulation of adhesion molecules.

This central and initiating EC-driven element of CM pathology is modulated by cytokines such as interferon-γ, TNF (previously called TNF-alpha) and lymphotoxin (previously called TNF-beta) (5). High levels of pro-inflammatory cytokines, in particular TNF, have been detected in the blood and the brain of patients who succumbed from CM as well as in models of ECM (29). Further supporting evidence has been provided for this scenario: *in vitro,* exposure to TNF induces upregulation of EC activation markers and IE pro-adhesion factors, and leads to increased IE binding on isolated human (30) or murine (31) brain microvascular cells.

IMMUNE CELL ACCUMULATION

Subsequent to the accumulation of IE and platelets, the intravascular accumulation of leucocytes is considered a key step in the development of ECM (32) and in some cases of HCM (3, 19, 23, 33). In ECM, this accumulation of host leucocytes in the brain microvasculature is correlated with the onset and severity of neurological signs and is thus considered to be largely responsible for neurological damage. While these immune subsets have been shown to mainly comprise monocytes, T cells and natural killer (NK) cells, their accumulation sequence and the relative pathological role of these and other cell subsets remain debated (**Figure 1**).

Numerous ECM studies suggest that accumulation of T cells (both $CD8^+$and $CD4^+$) cells in the brain of infected mice have major pathogenic roles. Supporting evidence has been obtained through anti-CD4 or anti-CD8 antibody depletion strategies (34, 35) just before or after the detection of neurological signs, or through the use of genetically deficient mice lacking functional $CD8^+$+ T cells (32, 36, 37). In these models, infected animals failed to develop ECM, or showed a reduction in the severity of the syndrome. RAG2-KO mice that have been shown to be resistant to ECM can develop this pathology following adoptive transfer of $CD8^+$ T cells isolated from PbA-infected ECM-susceptible WT mice. Furthermore, transfer of cells originating from perforin- or granzyme B-deficient mice does not result in ECM development, indicating an essential role for these cytotoxic molecules and supporting an effector function for $CD8^+$ T cells. On the other hand, $CD4^+$ T cell involvement in ECM pathogenesis appears to take on a helper role, as in the majority of studies and mouse strains used, their antibody-mediated depletion only prevents ECM development when performed before or early after infection (5, 34, 38, 39).

Evidence has documented a requirement for a concomitant presence of both T cells and IE for ECM development (40) as, in brains of mice infected with a parasite not causing CM, accumulation of $CD8^+$ T cells is still present but no IE sequestration is observed. Conversely, experimental depletion or blockade of T cell accumulation in ECM models correlates with a diminution of IE accumulation (38, 41). While the proportion of sequestered T cells that are parasite antigen-specific is not known, parasite antigen-specific T cells do sequester within brain microvessels during ECM (42). Blockade of the adhesion molecule LFA-1 in a therapeutic setting (i.e., after onset of clinical signs) has been shown to be effective in blocking ECM and promoting survival (43, 44). A recent study confirms these earlier observations and provides a potential mechanism by showing that late, combined administration of antibodies specific for VLA-4 and LFA-1 decreases $CD8^+$ T cell adhesion and numbers in cerebral blood vessels (39). Altogether, a current paradigm favors a mechanism involving high systemic levels of certain cytokines such as IFN-γ and TNF as well as binding of IE to brain vasculature. Both activate ECs which further increase binding of IE and allow recruitment and binding of T cells. These events participate in cross-presentation of malarial antigens accumulated on the luminal surfaces of cerebral blood vessels with parasite-specific $CD8^+$ T cells previously primed in the periphery. These $CD8^+$ T cells then cause local damage to the endothelium through cytotoxic mechanisms with ensuing BBB breakdown and neurological damage (30, 45).

While the view that T cells have a central role in ECM pathogenesis is undisputed, its translation to the human disease is less clear as there is little evidence for high numbers of $CD8^+$ T cells in HCM. In ECM, this $CD8^+$ T cell-centric model follows a neat sequence of events, but still presents some mechanistic gaps. Thus, several other cell types have been investigated and have been shown to be critically important. For example, local cross-presentation of parasite-derived antigens is unlikely to be performed by IE as they are devoid of MHC molecules. Several reports have suggested that activated ECs could perform this function after transfer of malarial antigens (30, 46–48). Others have repositioned myeloid cells in the spotlight, particularly intravascular sequestered monocytes that have long been reported in both HCM (3, 23, 33, 49, 50) and ECM (29). In ECM, we have shown, using intravital two-photon microscopy, that monocytes start accumulating in the brain several days prior to the onset of clinical signs (51). As the severity of the disease increases, slower rolling speed and increased adhesion of monocytes is directly observed in correlation with EC activation. Using high-dimensional flow cytometry we have calculated that at the onset of clinically-overt CM in mice, $CD11b^+$ cells constitute ∼80% of accumulating leukocytes in the brain vasculature at a time when T cells, B cells, neutrophils, DC, and NK cells remain numerically minor (52). Decreased ECM signs and pathology after global monocyte/macrophage depletion by clodronate liposomes (51) or after the abrogation of inflammatory monocyte recruitment by anti-CCR2 antibody (53) support a pathogenic role of these cells. The exact mechanisms by which inflammatory monocytes are involved in ECM pathogenesis still remain to be ascertained. Naturally, monocytes constitute a major source of cytokines and chemokines in CM pathogenesis and locally arrested monocytes could potentiate brain EC activation and recruitment of other leukocytes, notably $CD8^+$ T cells. We have confirmed this by showing the interdependent recruitment of monocytic cells and $CD8^+$ T cells to the central nervous system (51). Monocytes have the potential to differentiate into macrophage- or dendritic cell (DC)-like phenotypes. A recent study has reported that the majority of $CD11b^+$ cells whose numbers peak at the onset of ECM symptoms also express CD11c, F4/80 and high levels of MHCII. These authors conclude that

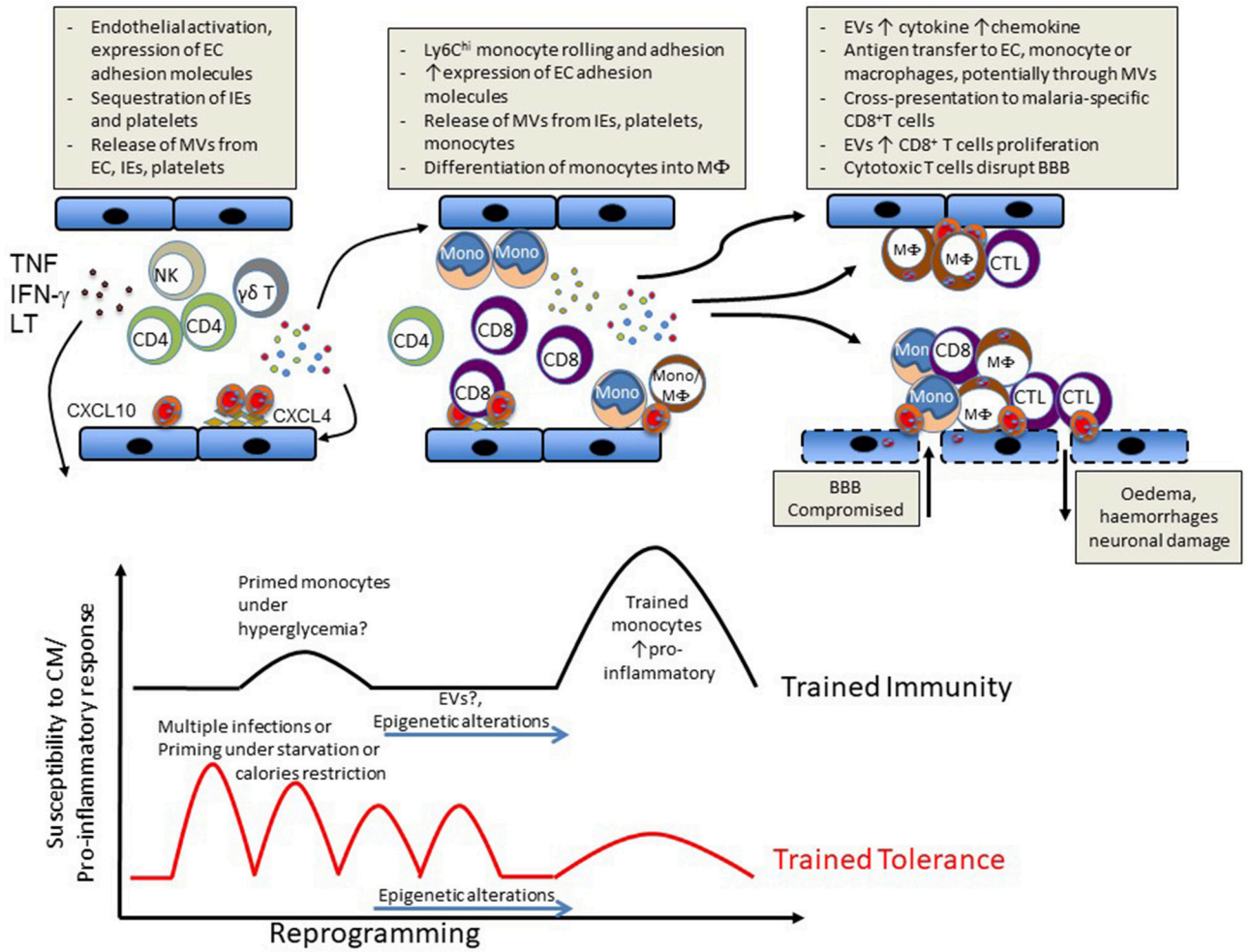

FIGURE 1 | Schematic representation of events likely to lead to CM **(Upper)** or to influence susceptibility vs. disease tolerance to CM **(Lower)**.

these are inflammatory monocytes that differentiate into splenic monocyte-derived DCs and are then subsequently recruited to the central nervous system (54). As opposed to resident macrophages and conventional DCs, a clear delineation between monocyte-derived DCs and monocyte-derived macrophages is still hotly debated, as extensively reviewed elsewhere (55). Recently, we have applied multiparametric high dimensional analysis using t-distributed stochastic neighbor embedding (tSNE) to further characterize the cerebral $CD11b^{+}$ populations in ECM. This has allowed us to identify a population of non-resident $Ly6C^{lo}$ cells with a monocyte/macrophage phenotype as the most prevalent population in the CM brain vasculature at peak disease. Importantly, we have further demonstrated that these monocytes derive from $Ly6C^{hi}$ precursors and are pathogenic. Indeed, immune-modulatory particles (IMP)—previously shown to specifically target $Ly6C^{hi}$ monocytes via induction of their sequestration in the spleen (56)—dramatically reduce $CD11b^{+}Ly6C^{lo}$ numbers in the brain and protect against cerebral and pulmonary lesions, with evidence of a down-modulation of immunopathology (52). Moreover, we have shown that when used in combination with established anti-malarial drugs, IMP treatment is highly effective (88% survival) even when given after neurological signs are present.

In addition to their protective role during malaria (57), γδ T cells have also been suggested as contributors to pathogenesis as they were found in patients (58), to be one of the predominant sources of cytokines and chemokines associated with severe malaria (50) Protection from ECM development following antibody-mediated depletion of γδ T cells supports this notion (59). However, γδ T cell depletion only prevents ECM development when performed before or early post infection, and such an effect is not observed in mice genetically deficient in γδ T cells.

Subsets of innate lymphoid cells (ILC) have been suggested to have distinct roles in CM. While expansion of ILC2 prevents ECM via a mechanism dependent on M2 macrophages and T regulatory cells (60), there is evidence that ILC1, through their cytotoxic NK subset, are involved in pathogenesis (61). However,

once again, NK cell depletion prevents ECM, but only in a prophylactic setting.

Thus, while targeting many immune cell types prevents the development of ECM, it is only the direct targeting of $CD8^+$T cells or of monocytes that has shown therapeutic effectiveness. The highly effective late stage success of the combination treatment with IMP and established anti-malarial drugs (52) highlights the novel potential of immunomodulatory targeting of innate immune cells in addition to $CD8^+$ T cells in severe malaria, with a potential avenue for human translation where the role of $CD8^+$ T cells is still unclear.

EXTRACELLULAR VESICLES: BOTH IMMUNOMODULATORS AND EFFECTORS OF PATHOLOGY

A hint of a role of membrane microvesicles (originally called microparticles) in immunopathology was adduced from experiments in which we attempted to understand the effector mechanisms of TNF in microvascular damage. Among these was the demonstration that TNF dose-dependently enhanced the release of microvesicles by endothelial cells (62). In parallel, microvesicles were found to be overproduced in the mouse model of CM. The majority of these microvesicles were from platelet origin, and seemed to be of pathogenic importance. Treatment of infected mice with an anti-CD40L mAb reduced microvesicle levels and thrombocytopenia, suggesting that CD40L is the main effector of malarial-induced thrombocytopenia. A role for platelet caspases *in vivo* was demonstrated by treatment of infected mice with the caspases inhibitor ZVAD-fmk, which reduced CM-associated mortality (28).

We subsequently found high microvesicle levels in the plasma of Malawian patients with CM. Remarkably, these high plasma levels were not seen in patients with severe malarial anemia, suggesting a relationship between microvesicle overproduction and the neuropathology (18). Since then, evidence has emerged that an important part of the immunopathological reaction is the release of extracellular vesicles, notably microvesicles (6) (**Figure 1**). Indeed, an effector role for microvesicles in immunopathogenesis is supported by the following evidence: (1) microvesicles can alter endothelial cell phenotype and function (63). (2) microvesicles are strong pro-inflammatory elements (64). (3) interfering with microvesicle binding to target cells reduces their activation (65), and (4) passive i.v. transfer of purified microvesicles exacerbates the disease in malarial-infected mice, and *in vitro* generated endothelial microvesicles even trigger CM-like lesions in naïve recipients (13).

We have found that ABCA1 deletion, which perturbs microvesicle release (66), fully protects mice against CM (67). Furthermore, blocking microvesicle production pharmacologically is beneficial for endothelial integrity (68, 69) and can prevent mortality due to ECM (70). Of note is that not only host-derived microvesicles appear important in CM: we have also shown that microvesicles released by malaria-infected erythrocytes *in vivo* are strongly pro-inflammatory in ECM, as evidenced by increased TNF production and CD40 expression on macrophages (64). This finding is not restricted to the experimental mouse model: plasma levels of erythrocytic microvesicles also are elevated in CM patients (71, 72).

Additionally, microvesicles have the potential to participate in antigen presentation, to express accessory molecules (48) and to amplify T cell proliferation, a property that we also had demonstrated in the context of TB (73). These results, together with those of Ramachandra et al. (74), suggest that microvesicles can be immunomodulatory elements, in addition to their effector roles in immunopathogenesis.

The involvement of microvesicles in CM remains incompletely understood because flow cytometry presents some limitations for their quantitation and characterization (75). Plasma microvesicles appear to carry discrete sets of miRNAs in relation to CM development (76). Malaria parasite themselves directly lead to the release of EV that contain small regulatory RNAs (77). Other aspects indicating a wider involvement of extracellular vesicles in malarial pathogenesis have been recently reviewed (78, 79).

IMMUNOMETABOLISM AND CM DEVELOPMENT

The switch from oxidative phosphorylation to aerobic glycolysis and glutaminolysis was first described by Otto Warburg close to a century ago in the case of rapidly proliferating cancer cells (80). Since then it has become clear that reprogramming of transcriptional but also metabolic programs is a required intrinsic cellular adaptation to adjust tissue function in response to stresses. Injury and/or repair responses have been shown to drive metabolic changes to accommodate increased or specific demands. Proper organismal metabolic state adjustment is considered critical in maintaining disease tolerance while maladaptation is seen as contributing to pathology or long-term sequelae. These metabolic changes can be triggered by a plethora of signals such as certain cytokines or hypoxia. The mechanistic target of rapamycin complex 1 (mTORC1), a protein kinase complex expressed in most eukaryotic cells, is considered a critical signaling integrator that links these stimuli as well as nutrient sensing to the coordinated regulation of cellular metabolism (81, 82).

Following a seminal paper demonstrating that the co-stimulation of T cells with anti-CD28 increased glucose uptake and glycolysis (83), the same principles of metabolic reprogramming have been shown to be as critical in other immune cells in response to cytokines, antigens (84) or pathogen-associated molecular patterns (PAMPS) (85) and damage-associated molecular patterns (DAMPS).

In turn, the metabolic status at the cellular level affects and constrains cellular functional polarization, e.g., in key developmental steps or in pro- vs. anti-inflammatory polarization of immune cells. One of the mechanisms highlighted is the potential role of some metabolites as stabilizers for transcription factors such as in the case of succinate build up following OXPHOS disruption acting as an inflammatory molecular switch through stabilization of HIF1-α. This

realization has resulted in a partial re-definition and extension of the field of immunometabolism from well-known regulatory roles of the immune system on local and systemic metabolism (tissue immunometabolism) to the additional modulatory role of cell intrinsic metabolic pathways on immune functions (cellular immunometabolism) [reviewed in (86)].

For example, it has been increasingly appreciated that T cell activation results in metabolic reprogramming from oxidative phosphorylation (OXPHOS) to glycolysis to meet the increased energetic and biosynthetic demands for T cell expansion and effector functions (87, 88). It was also conceived that promotion of effector cell differentiation, memory recall response or, on the contrary, regulatory phenotypes can be achieved by shifting their metabolism through changes in the availability of specific nutrients such as glucose or short chain fatty acids (SCFAs) (89, 90). In the case of monocytes/macrophages, the common view is that glycolysis supports inflammatory phenotypes or is favored after activation (91), while OXPHOS, presumably for sustained energy production, is a feature of resting monocytes/macrophages or macrophages with anti-inflammatory phenotypes. However, recent developments imply a more complex situation as glucose metabolism is still required in both anti-inflammatory and inflammatory macrophages (92, 93) and OXPHOS can also drive inflammasome activation in inflammatory macrophages (94).

Significant metabolic changes such as hypoglycaemia and lactic acidosis as well as perturbations in amino acid metabolism have long been observed in severe malaria in both ECM (95–97) and HCM (98–100). High brain concentrations of lactate, alanine, and glutamine are present in mice developing ECM but not in mice resistant to ECM (95, 101). We have demonstrated that elevated lactate was not uniform but occurred at the site of immunopathology, and that malaria parasites were not the dominant source of elevated lactate (102). These metabolic changes are consistent with, and have been mainly attributed to the occurrence of hypoxia/ischemia that follows obstruction of brain microvessels as in the case of ECM, both types of oedema, namely cytotoxic, and vasogenic oedema, have been demonstrated (97).

However, the recent developments in our understanding of immunometabolism mentioned above have called for a reassessment of hypotheses on mechanisms leading to CNS metabolite changes during CM development. It had been observed for some time that dietary restriction could prevent development of ECM, although only in a prophylactic setting (103, 104). Several recent studies have provided mechanisms on how manipulation of metabolism could explain this phenomenon. In independent studies, Gordon et al. (105) and Mejia et al. (106) demonstrated that caloric restriction or treatment with rapamycin, an inhibitor of mTOR, blocked the development of ECM. Remarkably, another study (107) showed that manipulating the glutamine pathway using the glutamine analog 6-diazo-5-oxo-L-norleucine (DON) rescues mice from ECM even when administered late in the infection. While the mechanisms proposed for these observations relate to blocks in activated T cell metabolism, few changes were observed in the numbers of T cells and other leucocytes recruited to the brain, and thus effects on other immune or mesenchymal cells remain a possibility. Indeed, a more recent report has shown that inhibition of glycolysis using the competitive glucose analog 2-deoxy glucose [2DG] was protective in ECM, not through effects on parasitaemia, the extent of anemia, the degree of cerebral oedema, or neuroinflammation but rather through modulation of haemostasis (108). These recent results emphasize the contribution of altered metabolic regulation to ECM and, more generally, to malarial pathogenesis in mice. The exact mechanisms behind these effects, the main cellular players involved, as well as well as their validation in HCM still requires investigation.

Because metabolic reprogramming can occur in response to changes in nutrient and oxygen availability, and in response to cytokines or other immune receptor stimulation, it presents the opportunity to therapeutically target these mechanisms at different levels, such as through dietary intervention. For example, pre-exposure to a high-fat diet reduced ECM via a mechanism that involved antioxidants (109). Another exciting prospect is the potential role of EVs in mediating such metabolic reprogramming. EVs could modulate metabolic pathways by virtue of transfer of miRNAs, mRNAs, proteins, and packaged metabolites to target cells. Such mechanisms have been described in response to other challenges such as retemplating of hepatic metabolic pathways through muscle-derived exosomes following intense exercise (110). As developed above, MVs and, to a lesser extent, exosomes are an essential component of CM development in both ECM and HCM but such link between the release of MVs in CM and immunometabolism regulation remains to be investigated.

"INNATE IMMUNE MEMORY" AND TRAINED IMMUNITY IN CM DEVELOPMENT

"Trained immunity" is defined as the capacity of some innate immune cells such as monocytes/macrophages to display an enhanced or polarized immune response upon non-specific restimulation (111). Mechanistic studies have highlighted long-term epigenetic changes as being largely responsible for this phenomenon or the converse, the situation where a previous trigger leads to a decreased responsiveness to a subsequent immune challenge [reviewed in (112)]. These recent observations have cemented the long-proposed concept of "innate immune memory" (113, 114). The increased proinflammatory cell programs characterizing trained immunity or, on the contrary, "tolerised" innate immune phenotypes are associated with changes in cellular metabolism such as the metabolic shift from OXPHOS toward glycolysis occurring in *Candida albicans*-derived ß-glucan-induced trained immunity (111, 115).

An important recent discovery is the observation that metabolites such as succinate and fumarate can serve as modulators of epigenetic enzymes such as histone and DNA demethylases (116). Thus, the buildup of these TCA cycle metabolites or some derivatives such as aconitate (117) following OXPHOS disruption has been proposed to play a

major role in innate immune memory. Such a link between metabolic reprogramming and trained immunity has the potential to provide novel insights into the pathogenesis of several diseases. Therefore, ongoing research is seeking to understand how innate immune memory, in some cases via immunometabolic modulation, could provide enhanced protection against reinfection, heterologous benefits against unrelated pathogens or on the contrary increase risks of post infection host-mediated pathology and increased susceptibility to chronic inflammatory diseases.

While beyond the scope of this manuscript, a large number of epidemiological studies have shown that outcomes of malarial infections are influenced by age and previous exposure. Furthermore, in specific resistant or susceptible ethnic groups where frequencies of single nucleotide polymorphisms (SNPs) in classical malaria-resistance genes cannot explain interethnic differences, epigenetic modifications or different chromatin landscapes were observed on specific host gene promoters or host genes that provide resistance to or susceptibility to malaria (118–121). In this context, there is also growing evidence that parasite infection can induce a state of trained innate immunity as exemplified by the hyperresponsiveness to TLR ligand stimulation of peripheral blood mononuclear cells (PBMCs) from patients with acute febrile disease *in vitro* (122). Additionally, higher numbers of IFN-γ producing PBMCs obtained following *P. falciparum* peptide stimulation in children suffering from malaria were correlated with significantly lower rates of malaria reinfection (123, 124). Approaches using controlled human malarial infections or vaccine trials have reported the induction of memory-like NK cells able to produce IFN-γ upon recall with malarial antigen or vaccine peptides. (125–127). A recent study has shown that PBMCs previously exposed to *P. falciparum* display an enhanced response to subsequent TLR2 stimulation and that this hyperresponsiveness correlated with increased methylation at specific proinflammatory and immunometabolic promoters. Importantly, these epigenetic modifications were also seen in Kenyan children infected with malaria (121). To our knowledge, there is no report of a direct link between trained immunity and CM. However, it is reasonable to hypothesize that such enhanced response training, although beneficial in fighting the parasite and assisting adaptive immunity, may increase the risk of severe disease upon reinfection. On the other hand, multiple episodes of malarial infection could instead induce tolerance to subsequent infection through modulation of this innate immune memory (**Figure 1**).

Much work remains to be done in order to integrate the interactions between immunologic signals, metabolic changes and epigenetic modifications with long-term changes in "innate immune memory" in response to malaria. Deciphering these mechanisms could have far reaching implications. It could help predict the risk of developing CM in individuals with previous (including *in utero*) malarial exposure. Modulation of trained immunity would also influence the clinical development of rationally designed malaria vaccines. Finally, this knowledge may help re-train innate cells during or before the disease to prevent severe complications. One could imagine that EVs may provide a way to achieve this by shuttling a designed cargo of miRNAS, epigenetic modifying enzymes or metabolites.

CONCLUSION AND FUTURE DIRECTIONS

The ECM model has produced a wealth of information on CM pathogenesis in mice with the aim to find an adjunctive therapy for HCM but its validity has been questioned. However, several investigators have provided a critical and evidence-based defense of this model (17, 128–131) and from knowledge gained from it, numerous laboratories have tested preclinical therapeutic interventions. Many have demonstrated efficacy at blocking the development of ECM but disappointingly, in a majority of cases, this was only found when administered before or early post infection and prior to the onset of clinical neurological signs. Therefore, only a few can justify, as therapies, a large-scale evaluation in HCM. A rare case of efficacy of treatment even when administered after the onset of clinical signs in the ECM model is the injection of IMP (52). The only other studies to date having demonstrated treatment efficacy after the onset of ECM have targeted $CD8^+$ T cell binding to endothelial cells, and finally, immunometabolism. These successes have renewed hopes that the mouse model of ECM will continue to bring novel ideas/concepts which then will need to be confirmed or infirmed in HCM. For example, the characterization of retinal pathology in ECM (132) and (133), which was followed by the demonstration of its usefulness in HCM (134–137) (and many others) or MRI findings of brain alterations, originally described in 2005 for ECM (97), and followed by similar findings in HCM (138, 139).

In addition, it should be emphasized that the massive reduction in malaria burden achieved in the last two decades was mostly a result of prevention strategies rather than treatment. In this context, knowledge gained from ECM studies should not be seen as limited to the design of therapies but could also guide the expansion of our arsenal for HCM prevention. In particular, understanding changes in innate immune memory preconditioning and metabolic status in populations with high or low incidence of HCM could present opportunities for prevention through environmental or dietary interventions.

AUTHOR CONTRIBUTIONS

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HVEM and CD160: Regulators of Immunopathology During Malaria Blood-Stage

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CD8$^+$ T cells are key players during infection with the malaria parasite *Plasmodium berghei* ANKA (PbA). While they cannot provide protection against blood-stage parasites, they can cause immunopathology, thus leading to the severe manifestation of cerebral malaria. Hence, the tight control of CD8$^+$ T cell function is key in order to prevent fatal outcomes. One major mechanism to control CD8$^+$ T cell activation, proliferation and effector function is the integration of co-inhibitory and co-stimulatory signals. In this study, we show that one such pathway, the HVEM-CD160 axis, significantly impacts CD8$^+$ T cell regulation and thereby the incidence of cerebral malaria. Here, we show that the co-stimulatory molecule HVEM is indeed required to maintain CD8$^+$ T effector populations during infection. Additionally, by generating a CD160$^{-/-}$ mouse line, we observe that the HVEM ligand CD160 counterbalances stimulatory signals in highly activated and cytotoxic CD8$^+$ T effector cells, thereby restricting immunopathology. Importantly, CD160 is also induced on cytotoxic CD8$^+$ T cells during acute *Plasmodium falciparum* malaria in humans. In conclusion, CD160 is specifically expressed on highly activated CD8$^+$ T effector cells that are harmful during the blood-stage of malaria.

Keywords: cerebral malaria, CD8 T cells, co-inhibitory receptors, HVEM, CD160

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INTRODUCTION

The inflammatory response to blood-stage malaria is characterized by a strong Th1 polarization and T cell induced immunopathology. It has been shown that when C57BL/6J mice are infected with the parasite *Plasmodium berghei* ANKA (PbA), cytotoxic CD8$^+$ T cells do not contribute to the elimination of the parasite during blood-stage, but rather cause the disruption of the blood-brain barrier. *Plasmodial* antigens can indeed be cross-presented on activated brain endothelial cells (1) leading to the release of cytotoxic molecules and pro-inflammatory cytokines such as granzymes and IFNγ by T cells (2–5). This leads to the severe manifestation of experimental cerebral malaria (ECM) (5).

T cell function is tightly controlled by the integration of co-inhibitory and co-stimulatory signals. We have shown and so have others that the co-inhibitory receptors PD-1, CTLA4 and BTLA are induced during malaria. These co-inhibitory receptors play an important role in the regulation of CD4$^+$ T cell activation thus controlling immunopathology during the blood-stage (6–11). In contrast, during the liver-stage of malaria they restrict the protective function of CD8$^+$ T cells (12). Of note, the control of CD8$^+$ T cells during the blood-stage of the infection

and ECM remains to be fully understood. Dissection of the impact of different immunomodulatory receptors in T cell regulation is essential not only for our understanding of T cell biology but also for the therapeutic use of checkpoint inhibitors. This might allow us to dampen unwanted immune responses without lowering protection and to increase protection without the risk of overwhelming inflammation. We have previously described the protective function of BTLA antagonists in experimental cerebral malaria (7), yet a deeper understanding of the HVEM-network during malaria still represents a crucial step to develop and to broaden future therapeutic approaches. Besides BTLA, CD160, LIGHT, and LTα are known HVEM-ligands. LIGHT-HVEM interaction does not impact the development of experimental cerebral malaria, while local LTα drives cerebral pathology (13, 14). HVEM is an important co-stimulatory receptor expressed by T and B cells, endothelial cells and mast cells (15–18). HVEM-signaling enhances the expansion of T cells and is required for the persistence of memory T cells in bacterial and viral infections and in experimental settings of challenge with model antigen (19–22). Still its exact function in models of parasitic infections requires further investigation. We hypothesized that during malaria, HVEM via co-stimulation of $CD8^+$ T cells plays a critical role in the development of the cerebral symptoms. In addition, we also focused our attention on the HVEM-ligand CD160, which shares the binding region with BTLA. CD160 is described to be expressed by cytotoxic NK and $CD8^+$ T cells, NKT cells, a minority of $CD4^+$ T cells, γδ T cells, iIELs, ILC1, and mast cells (23–28). CD160 function on T cell regulation remains controversial as some reports describe CD160 to be a co-inhibitory molecule whereas others suggest that it exhibits a co-stimulatory function enhancing proliferation, inflammatory cytokine production and cytotoxic capacity (15, 29–32). In this study we addressed this unanswered question by generating CD160-deficient mice and analyzing the $CD8^+$ T cell profile during experimental cerebral malaria.

Our data demonstrate that HVEM is required to stabilize $CD8^+$ T effector cell populations during acute *P. berghei* ANKA (PbA) infection. Expression of CD160 is specifically induced in highly activated, cytotoxic $CD8^+$ T cells concurrently with the onset of cerebral symptoms. However, an aggravated T cell mediated pathology upon infection with PbA in $CD160^{-/-}$ mice suggests a co-inhibitory function of CD160. Importantly, we found CD160 expression by $CD8^+$ T cells not only in mice infected with PbA but also in patients suffering from acute malaria.

MATERIALS AND METHODS

Mice

C57BL/6J, $HVEM^{-/-}$ (33), $CD160^{-/-}$ (see section Generation of $CD160^{-/-}$ Mice), OT-1CD45.2CD90.1 and $HVEM^{-/-}$ OT-1CD45.2 (from now on referred to without congenic markers) mice were bred in the animal facility of the Bernhard Nocht Institute for Tropical Medicine and maintained in a specific pathogen-free facility. Age-matched (7–8 weeks of age) female mice were used. All experiments with mice were approved by the City of Hamburg Office for Consumer Protection (56/13; 32/15).

Human Samples

All experiments performed with human peripheral blood were approved by the Ethics Committee of the Medical Association Hamburg. Male malaria patients at an average age of 44 (range 26–69 years) with a travel history in malaria endemic regions and confirmed *Plasmodium falciparum* positive blood smears (Ethics Approval PV4539) were recruited between October 2015 and August 2016 at the University Medical Center Hamburg-Eppendorf. The patient samples include anonymous acquired discarded tissue samples of the diagnostic lab at the Bernhard Nocht Institute for Tropical Medicine. Analysis of blood samples was performed at the day of transmission to hospital or up to three days after treatment. Five patients had a parasitemia lower than 1%, three lower than 3% and one patient showed hyperparasitemia (8%) at the transmission to hospital. Members of the Bernhard Nocht Institute for Tropical Medicine were recruited as healthy controls. Control patients with chronic hepatitis B virus infection (HBV), autoimmune liver diseases (primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH) were recruited at the liver outpatient clinics at the University Medical Center Hamburg-Eppendorf and gave written informed consent (Ethics Approval PV4081; PV5661). In total, 10 malaria patients and 9 healthy controls were included in the study. Informed consent was obtained from all individuals included.

Flow Cytometry of Human Cells

Peripheral blood samples were first stained for surface epitopes [αCD8 AF700 (53-6.7), αCD28 BV510 (CD28.2), αCD69 FITC (FN50), αCD160 PE-Cy7 (By55), αPD-1 PerCP-Cy5.5 (EH12.2H7) from BioLegend] including a live/dead staining reagent (LIVE/DEAD Fixable Blue Dead Cell Stain Kit for UV excitation from ThermoFisherScientific) for 30 min at 4°C. Afterwards, lymphocytes were fixed and red blood cells (RBCs) were lysed (1-step Fix/Lyse solution from eBioscience). Fixation and permeabilisation of cells was performed using the Foxp3/Transcription Factor Buffer Set (ThermoFisherScientific) according to the manual. To block unspecific binding of antibodies, cells were incubated for 10 min at 4°C with CohnII and subsequently, antibodies directed against intracellular epitopes (GzmB AF647 (GB11), CTLA-4 PE (L3D10), Ki67 AF488 (Ki-67), CD3 APC-Cy7 (HIT3a) from BioLegend, Perforin BV421 (δG9) from BD) were added and further incubated for 20 min at 4°C. Samples were recorded using the LSRII (BD) and analyzed using the FlowJo X 10.0.7r2 Treestar software. Gates were set according to fluorescence minus one (FMO) controls. Gating strategy is depicted in **Supplementary Figure 5**.

Adoptive, Competitive T Cell Transfer

$CD8\alpha^+$ T cells were isolated from total splenocytes of OT-1CD45.2CD90.1 and OT-1CD45.2x$HVEM^{-/-}$ by MACS ($CD8\alpha^+$ T Cell Isolation Kit, mouse from Miltenyi), labeled with proliferation dyes (Cell Proliferation Dye eFluor™ 450 or 670 from eBioscience) according to the manual. Both $CD8^+$ T cell populations were mixed in a 1:1 ratio and transferred i.v. in C57BL/6JCD45.1 mice. Mice were infected on the same day with

Pb-OVA (see 2.4). Five days after transfer and infection, blood and splenocytes were isolated and analyzed.

Plasmodium Infections

Plasmodium berghei, Plasmodium yoelii, and *Plasmodium berghei-OVA* (34) parasites were stored in 0.9% NaCl, 4.6% sorbitol and 35% glycerol in liquid nitrogen. In order to increase viability of the parasites, they were passaged once in C57BL/6J mice. Subsequently, fresh blood of passage mice was used to transfer 1x 10^5 infected red blood cells/200 µl PBS i.p. in mice used for experiments.

Cerebral symptoms were scored according to the following scheme: 0= no symptoms; 1= decreased activity, deceleration; 2= ataxia; 3= weight loss≥ 20% or convulsions, strong ataxia; 4= coma; 5= death. If a score of ≥3 was reached, mice were euthanized in order to avoid unnecessary suffering.

Flow Cytometry of Murine Cells

Splenocytes were isolated as described previously (35). Blood was collected using heparinized syringes and washed in PBS. Red blood cells were lysed by incubation in lysis buffer (10 nM tris pH 7.2, 0.15 M ammonium chloride). Brain tissue was cut into small pieces, suspended in PBS and filtered through a 70 µm sieve. In order to obtain intestinal intraepithelial lymphocytes, the intestine was dissected from the stomach to the caecum and placed at a PBS drained tissue. At this stage, Peyer's patches and fatty tissue were removed, the intestine opened longitudinally, gently washed in PBS and cut into 1 cm pieces. The pieces were digested in 20 ml digestion medium (30 mM EDTA, 10% FCS in PBS) for 30 min at 37°C. To improve the suspension of intraepithelial lymphocytes, the tubes were shaken regularly. After 30 min, the tubes were vigorously shaken 10x and the suspension filtered through a tea sieve. The washing step was repeated twice by adding 10 ml digestion medium to intestine pieces followed by 10x shaking and filtration. IIEL were separated from the mucus using 37% percoll.

Cells were stained with the LIVE/DEAD Fixable Blue Dead Cell Stain Kit, for UV excitation (ThermoFisherScientific) and subsequently stained with antibodies for surface epitopes (CD3 AF488 (145-2C11), CD8 AF700 (53-6.7), CD19 PE (1D3), CD28 APC (37.51), CD44 PE-Cy7 (IM7), CD107a BV421 (1D4B), CD160 PerCP-Cy5.5 (7H1), PE-Cy7 (RMPI-30) from BioLegend, CD8 V450 (53-6.7), CD11a PE/V450 (M17/4), BTLA PE (6F7), γδ FITC (eBioGL3), HVEM APC (LH1), KLRG1 eF780 (2F1) eBioscience, CD4 V500 (RM4-5), PD-1 PE-Cy7 (PK136), TCRβ APC (H57-597) BD, CD45 FITC from Caltag). After fixation and permeabilisation (Foxp3/Transcription Factor Buffer Set from ThermoFisherScientific) intracellular staining was performed (GzmB AF647 (GB11), Ki67 AF488 (Ki-67) from BioLegend). Samples were recorded using the LSRII and analyzed using the FlowJo X 10.0.7r2 Treestar software. Gates were set according to fluorescence minus one (FMO) controls. Gating strategy is depicted in **Supplementary Figure 4**.

Staining of mRNA for Flow Cytometry

In order to stain mRNA in addition to proteins for analysis by flow cytometry the PrimeFlow RNA Assay Kit from ThermoFisherScientific was used according to the manual provided. The probes included were directed against *Ifng*, *CD160,* and *β-Actin* (positive control) mRNA.

PbA-Specifc *in vitro* Stimulation

Stimulation of PbA specific $CD8^+$ T cells was performed with a mixture of three MHCI restricted PbA peptides [Pb1: SQLLNAKYL, Pb2: IITDFENL and F4: EIYIFTNI from Jerini Biotools (5)]. In order to pulse Hepa1-6 cells with peptides, complete RPMI (5% FCS, 1% L-Glutamine, 0.5% Gentamycine) was supplemented with 1 µg/ml of each peptide. After 3–4 h the medium was removed and cells were washed with PBS. Subsequently, $CD8^+$ T cells were added in complete RPMI and co-cultured with the peptide-presenting cells. For detection of cytokines from cell culture supernatants, sandwich ELISAs (RnD) was performed according to the manual.

Cytotoxicity Assay

The cytotoxic effector function of $CD8^+$ T cells was analyzed by a flow cytometry based assay (LIVE/DEAD cell mediated cytotoxicity Kit from ThermoFisherScientific) according to the manual. First, target cells, namely splenocytes from naïve mice, were pulsed with 1 µg/ml of Pb-peptides [Pb1: SQLLNAKYL, Pb2: IITDFENL and F4: EIYIFTNI from Jerini Biotools (5)] for 3 h at 37°C. Afterwards, cells were washed, diluted to 1x10^6 cells/ml and stained with 0.25 µl/ml Dioctadecyloxacarbocyanine Perchlorate (DIOC) for 20 min in PBS. In order to remove DIOC from the supernatant, cells were washed twice in PBS and suspended in complete medium to a concentration of 1×10^6 cells/ml. Second, effector cells from PbA infected mice at day 6 p.i. were sorted for $CD44^{hi}CD160^{+/-}$ cells and diluted to a concentration of 7.7×10^5 cells/ml. To detect dead cells, a 1 µg/ml propidium iodide (PI) solution in complete medium was used. Samples containing 1×10^5 effector cells, 1×10^4 target cells and PI solution were cultured 2 h before analysis.

Generation of $CD160^{-/-}$ Mice

$CD160^{-/-}$ mice were generated using the CRISPR/Cas9 technology. The target region for gene editing was chosen using the online tool CHOPCHOP (36). The following oligos consisting of the T7 promotor and the target region were used: fw GAAATTAATACGACTCAC TATAGGGAGAGCACAAGAAAGACGAAGCTGGT TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC; rev AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGAT AACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCT. Oligos were amplified by PCR, purified by agarose gel electrophoresis and extracted with the Qiaex II Gel Extraction Kit (Quiagen) according to the manual. The purified PCR products were used as templates for transcription into RNA. sgRNA and Cas9 protein were injected into cytoplasm and pronucleus of one-cell staged C57BL/6 mouse embryos and implanted into foster mothers. The heterozygous offspring was used as founder animals and crossed with C57BL/6J mice. Two knockout lines derived from different founder animals were bred and characterized. Because both lines behaved alike, only one was chosen for further experiments. Genotyping of mice

was performed by restriction-length fragment polymorphism (RLFP). Tissue samples were lysed and genomic DNA sequences consisting of the target region were amplified by PCR using the lysate as a template. Subsequently, PCR products were digested by an enzyme specific for the wild type sequence. Hence, wild type DNA is digested into two smaller fragments, while modified DNA remains at the original size.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 5.0b. The statistical significance between the two groups was calculated using the two-tailed Mann-Whitney test while correlation was calculated using the Spearmann Correlation test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

HVEM Controls CD8$^+$ T Cell Persistence During Malaria Blood-Stage

The role of co-stimulatory signaling by HVEM on proliferation of CD8$^+$ T cells was first evaluated using antigen-specific *in vitro* stimulation of wild type (WT) OT-1 and HVEM$^{-/-}$ OT-1 CD8$^+$ T cells. In this experimental setting, no differences in the proliferation of WT and HVEM$^{-/-}$ OT-1 CD8$^+$ T cells could be observed (**Figure 1A**). Next, we analyzed not only the proliferative capacity, but also the persistence of CD8$^+$ T cells during an acute *P. berghei* ANKA (PbA) infection by a competitive, adoptive T cell transfer assay in which WT OT-1 and HVEM$^{-/-}$ OT-1 CD8$^+$ T cells, labeled with different proliferation dyes, were mixed in equal proportions and transferred into WT hosts. Concomitantly, mice were infected with *P. berghei*-OVA (Pb-OVA). In this experimental setting, both CD8$^+$ T cell populations that can be tracked according to their different congenic markers (both T cell populations: CD45.2$^+$; WT OT-1: CD90.1$^+$; HVEM$^{-/-}$ OT-1: CD90.1$^-$; recipient: CD45.1$^+$CD45.2$^-$) are exposed to the same inflammatory environment and parasitemia level, thus specifically allowing the analysis of the HVEM function. Of note, both WT OT-1 and HVEM$^{-/-}$ OT-1 T cells were able to completely dilute the proliferation dye by day 5 post infection (p.i.) (**Figure 1B**). However, WT CD8$^+$ T cells persisted longer during Pb-OVA infection after adoptive T cell transfer compared to the HVEM$^{-/-}$ CD8$^+$ T cell counterpart (**Figure 1C**). Taken together, co-stimulation by HVEM is not required for initial expansion of effector cells, but crucial for their maintenance even during the first 5 days of blood-stage malaria.

Cerebral Malaria Development During PbA Infection Is Dependent on HVEM Engagement on CD8$^+$ T Cells

Infection of C57BL/6 mice with PbA causes disruption of the blood-brain barrier by cytotoxic CD8$^+$ T cells (5). When we examined the CD8$^+$ T cell pool in WT and HVEM$^{-/-}$ mice infected with the parasite, in line with our adoptive transfer experiment results (**Figure 1C**), we found significant reduction in CD8$^+$ T cell numbers in the blood of HVEM$^{-/-}$ mice compared to the control counterpart (**Figure 2A**). However, in contrast to OT-1 cells, which were used for the adoptive T cell transfer experiment, the pool of CD8$^+$ T cells in WT and HVEM$^{-/-}$ mice represents PbA specific and unspecific, naïve CD8$^+$ T cells. In order to analyze CD8$^+$ T effector cells, we examined the expression of the activation marker CD44 and the marker for antigen-experienced cells CD11a (37–39). In contrast to spleen and blood, where only a fraction of CD8$^+$ T cells co-expressed these markers, all brain-infiltrating cells were CD44^{hi} and CD11a$^+$. Thus, we considered this marker combination suitable for the detection of effector cells (**Figure 2B**). We have previously shown that the co-inhibitory receptor and HVEM-ligand BTLA is expressed on both activated and naïve CD8$^+$ T cells (7). In contrast to BTLA, we found that CD160, another HVEM-ligand sharing the binding region with BTLA, is selectively expressed on CD8$^+$CD44^{hi}CD11a$^+$ T cells, but not on naïve T cells (**Figure 2C**). Surprisingly, we observed a reduced frequency of CD8$^+$CD160$^+$ T cells in the blood of PbA infected HVEM$^{-/-}$ mice compared to the WT counterpart, and a diminished percentage of activated (CD8$^+$CD44^{hi}) and antigen-experienced (CD8$^+$CD11a$^+$) CD8$^+$ T cells (**Figure 2D**). Considering the detrimental role of CD8$^+$ T cells in the development of cerebral malaria, we thus monitored WT and HVEM$^{-/-}$ mice for the progression of cerebral symptoms. Indeed, PbA infected HVEM$^{-/-}$ mice developed less severe symptoms in terms of neurological defects compared to WT mice (**Figure 2E**). Interestingly, even though the total number of CD8$^+$ T cells in the brain was unchanged (**Figure 2F**), the frequency of CD8$^+$CD160$^+$ T cells was substantially reduced in HVEM$^{-/-}$ mice (**Figure 2F**) compared to WT controls. These findings show that HVEM is essential for regulating the magnitude of CD8$^+$ T effector cell activation, mirrored by the reduced expression of CD160, thereby controlling the development of cerebral malaria.

CD160 Characterizes Highly Activated and Cytotoxic CD8$^+$ T Cells

In light of our results on the selective expression of CD160 on CD8$^+$CD44^{hi}CD11a$^+$ T cells, their strong reduction in frequency and concurrent reduction of pathology in HVEM$^{-/-}$ mice, we further characterized the signature profile of CD8$^+$CD160$^+$ T cells. First, we observed that CD8$^+$CD160$^+$ T cells were enriched for markers of proliferation (Ki67), cytotoxicity (GzmB), degranulation (CD107a), and differentiation (KLRG1, PD-1) compared to CD8$^+$CD160$^-$ T cells, independently of whether they were derived from WT or HVEM$^{-/-}$ mice (**Figure 3A**). Second, expression of KLRG1 was lower in CD8$^+$CD160$^+$ T cells of HVEM$^{-/-}$ mice than in WT control. These data, together with the fact that a reduced frequency of CD8$^+$CD160$^+$ T cells have been observed in HVEM$^{-/-}$ mice (**Figures 2D,F**) and support the hypothesis that HVEM signaling is required for persistence of a population of terminally differentiated CD8$^+$ T cells during PbA infection.

In order to provide evidence of the actual functionality of CD8$^+$CD160$^+$ T cells, we devised *in vitro* assays in which CD8$^+$CD44^{hi}CD160$^+$ T cells from PbA infected mice (d 6

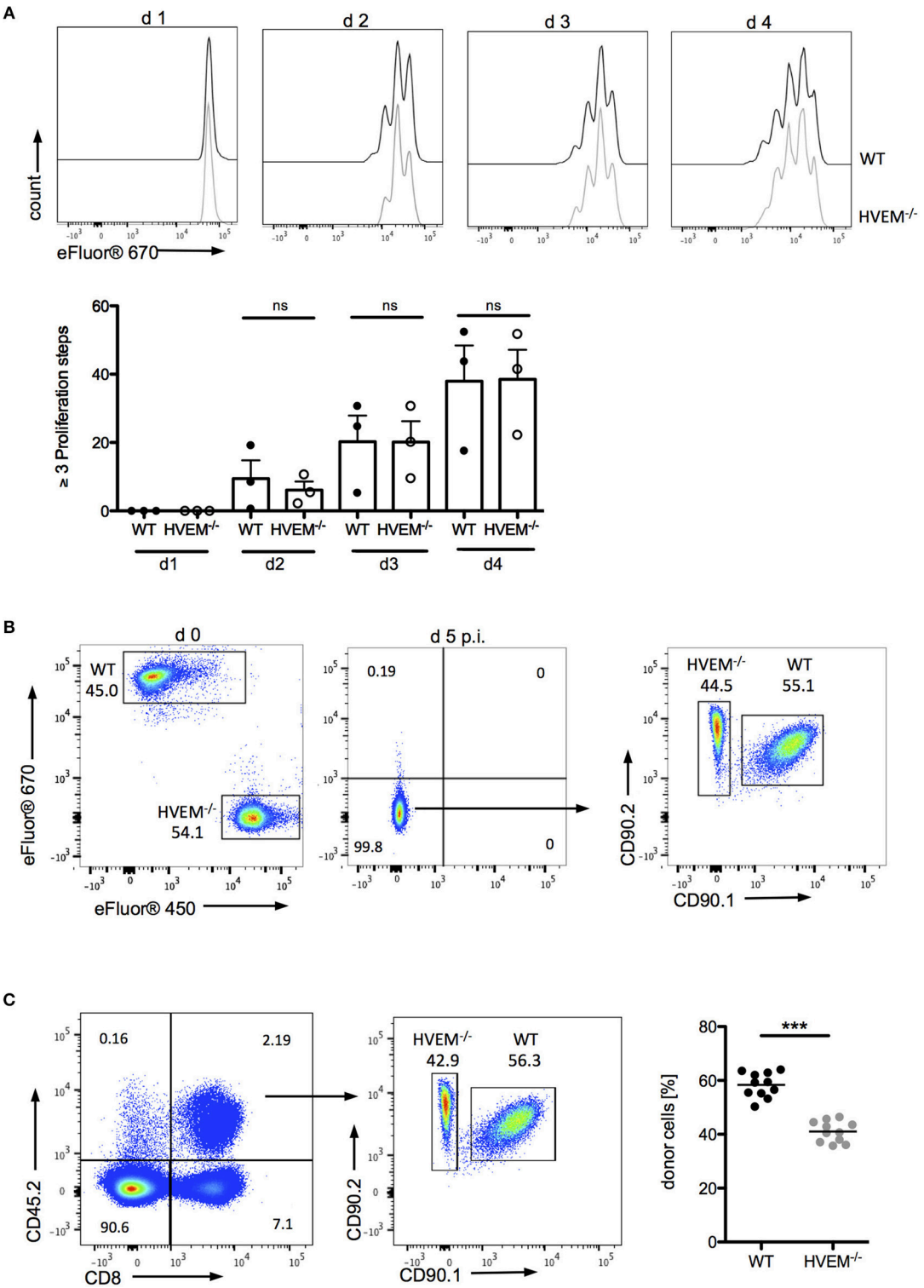

FIGURE 1 | HVEM is required for CD8$^+$ T cells persistence during malaria blood-stage. **(A)** Splenocytes of WT OT-1 or HVEM$^{-/-}$ OT-1 mice were labeled with the proliferation dye eFluor670 and stimulated for 1–4 days with the MHCI-restricted peptide SIINFEKL. Representative histograms are shown. Biological replicates from one experiment, which is representative of two independent experiments, are reported. The means ± SEM are depicted, each dot represents one mouse. For adoptive transfer assays, WT OT-1 and HVEM$^{-/-}$ OT-1 CD8$^+$ T cells were labeled with eFluor670 (WT) or eFluor450 (HVEM$^{-/-}$) proliferation dyes, respectively and transferred in a 1:1 ratio into a C57BL/6 host, which was concomitantly infected with Pb-OVA. At d 5 p.i. proliferation and persistence of transferred CD8$^+$ T cells was analyzed by **(B)** dilution of fluorescent dyes and **(C)** frequency of cells expressing the congenic markers CD45.2/CD90.2. Representative plots are depicted. Data were pooled from three independent experiments performed with 2–5 mice/group (total $n = 11$). ***$p < 0.0001$.

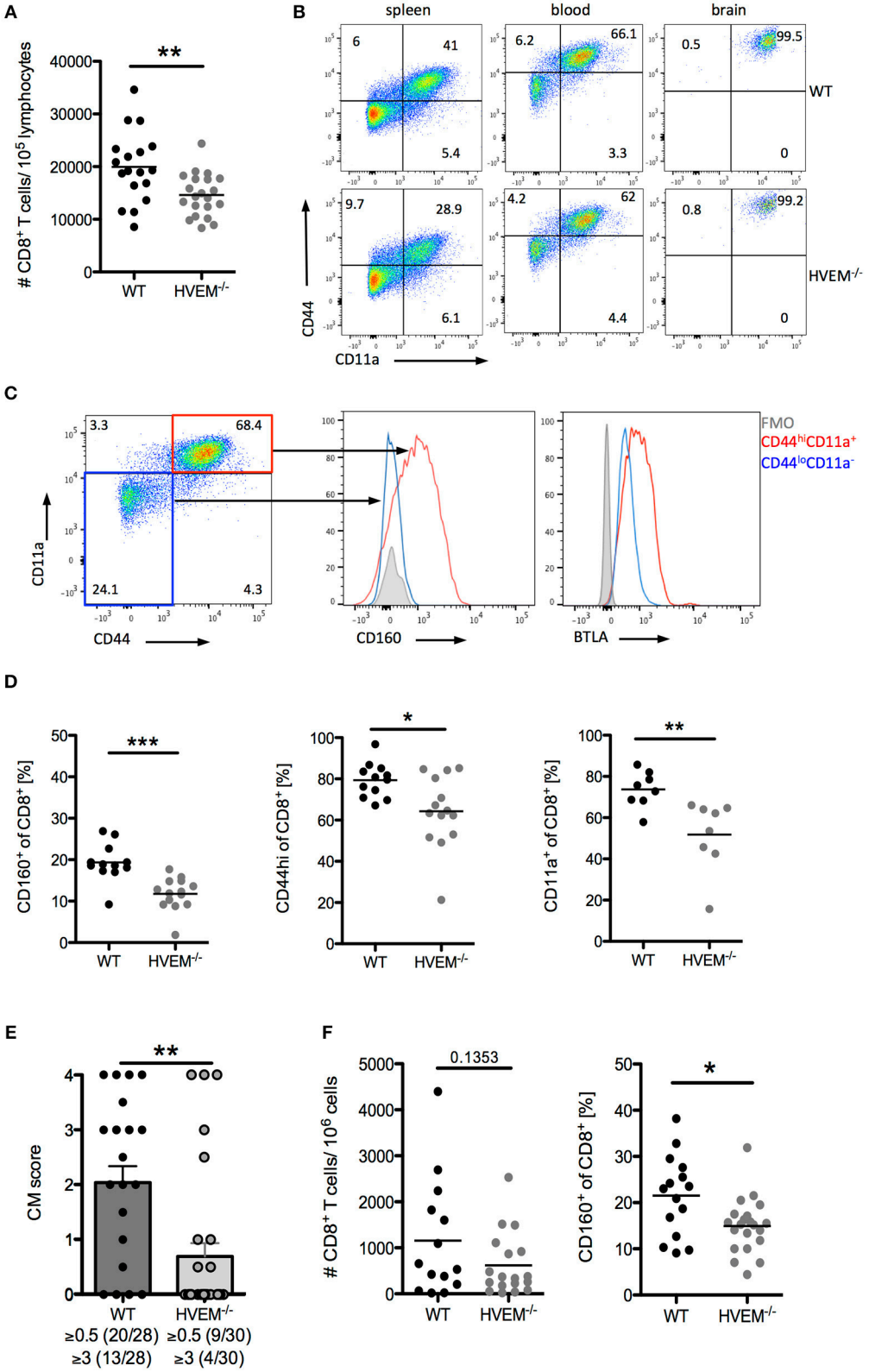

FIGURE 2 | HVEM engagement augments the development of cerebral malaria. Flow cytometric analysis of $CD8^+$ T cells in WT and $HVEM^{-/-}$ mice at d 6 post PbA infection. **(A)** Number of $CD8^+$ T cells in the blood. Pooled data of 5 independent experiments performed with 3–5 mice/group (WT $n = 18$, $HVEM^{-/-}$ $n = 21$).

(Continued)

FIGURE 2 | (B) Representative dot plots of CD44 and CD11a staining of $CD8^+$ T cells derived from spleen, blood or brain of wild type or $HVEM^{-/-}$ mice. **(C)** Expression of CD160 or BTLA within the $CD44^{lo}CD11a^-$ (blue) or $CD44^{hi}CD11a^+$ (red) cell population. **(D)** Frequency of $CD160^+$, $CD44^{hi}$ or $CD11a^+$ cells in the blood gated on $CD8^+$ T cells. Data were pooled from 2 (CD11a) or 3 (CD44, CD160) independent experiments performed with 3–6 mice/group (CD11a: WT $n = 8$, $HVEM^{-/-}$ $n = 8$; CD44: WT $n = 12$, $HVEM^{-/-}$ $n = 14$; CD160: WT $n = 12$, $HVEM^{-/-}$ $n = 14$). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. **(E)** Severity of cerebral symptoms was monitored in WT and $HVEM^{-/-}$ mice at d 6 p.i. Data were pooled from 8 independent experiments including 3–6 mice/group. $^{**}p = 0.0014$. **(F)** Number of brain-infiltrating $CD8^+$ T cells and frequency of $CD160^+$ T cells within the $CD8^+$ population. Data were pooled from 4–5 independent experiments including 2–5 mice/group (#CD8: WT $n = 14$, $HVEM^{-/-}$ $n = 18$; CD160: WT $n = 15$, $HVEM^{-/-}$ $n = 21$). $^*p = 0.0107$.

p.i.) were FACS sorted and analyzed for their ability to kill target cells and to produce the pro-inflammatory cytokine IFNγ. $CD8^+CD44^{hi}CD160^-$ T cells from PbA infected mice and $CD8^+CD44^{hi}$ T cells from naïve mice were used as internal control. In line with the difference in the cytotoxic characteristic observed (**Figure 3A**), $CD160^+$ cells were more effective in killing target cells, compared to the similarly activated ($CD44^{hi}$) $CD160^-$ counterpart (**Figure 3B**). Additionally, $CD160^+$ cells showed a trend to produce higher amounts of IFNγ compared to the control cells. However, no statistical significance was reached, which might be due to inter experimental variations (**Figure 3C**). Taken together, these data identify CD160 as signature of $CD8^+$ T cell with high killing capacity and IFNγ production in the context of blood-stage malaria.

$CD8^+CD160^+$ T Cells Are Associated With Cerebral Pathology

The phenotype and functional properties of $CD8^+CD160^+$ T cells suggest their involvement in cerebral pathology. Hence, we further correlated CD160 expression on $CD8^+$ T cells with the development of cerebral malaria. Using our protocol, severe cerebral symptoms in the PbA infection model occurred at day 6 p.i. with most of the mice succumbing to cerebral pathology between day 6 and 8 p.i. CD160 expression on $CD8^+$ T cells was thus analyzed early after infection (d 3 p.i,), before first symptoms occurred (d 5 p.i.), upon onset of severe symptoms (d 6 p.i.) and at the peak of T cell activation (d 7 p.i.). Strikingly, CD160 remained absent until d 5 p.i. and was clearly induced at the peak of pathology (days 6–7 p.i.) (**Figure 4A**). The highest frequency of $CD8^+CD160^+$ T cells was detected in blood and brain where the most tissue damage occurs, compared to the spleen (**Figure 4B**) where the parasitized RBCs are eradicated and T cells are activated (40). Importantly, the frequency of $CD8^+CD160^+$ T cells correlated with the severity of cerebral symptoms (**Figure 4C**).

Furthermore, we examined the induction of CD160 on $CD8^+$ T cells in a non-lethal model of malaria via injection of the parasite *Plasmodium yoelii* (PyNL). PyNL causes strong T cell activation ($CD44^{hi}CD11a^+$) compared to the lethal parasite strain (PbA). However, we observed reduced frequency of $CD8^+CD160^+$ T cells within the activated $CD8^+$ T cell population in non-lethally (PyNL) vs. lethally infected (PbA) WT mice (**Figure 4D**). Because mice are able to naturally clear PyNL parasite, we also analyzed CD160 expression on $CD8^+$ T cells after resolution of the infection (d 20 p.i.). Interestingly, $CD8^+$ T cells maintained stable CD160 expression up to day 20 p.i., even though their proliferation capacity is drastically reduced as shown by the down-regulation of the Ki67 epitope (**Supplementary Figure 1**).

Taken together, there are several findings that strongly suggest that CD160 contributes to $CD8^+$ T cell regulation in particular, in highly activated $CD8^+$ T cells, which are harmful in PbA infection: The kinetics of CD160 expression; tissue distribution of the $CD160^+$ cells; correlation of the frequency of $CD160^+$ cells and cerebral symptoms; and their reduction in the non-lethal plasmodial infection.

CD160 Restricts T Cell Mediated Immunopathology

A co-stimulatory role for CD160 has been previously suggested in NK cells and T cells in the context of allograft rejection, melanoma and lymphoma tumor models (41–43). Importantly, our data during malaria now strongly support the idea of CD160 as a critical regulator of $CD8^+$ T cytotoxicity and consequent detrimental function on the overall outcome of malaria. However, it seems reasonable that co-inhibitory receptors are induced especially on highly active cells in order to restrict T cell function and CD160 has already been described as a co-inhibitory receptor on human $CD4^+$ T cells (29). To address the role of CD160 in $CD8^+$ T cells in the PbA model, we generated $CD160^{-/-}$ mice using the CRISPR/Cas9 technology on C57BL/6 background (**Supplementary Figure 2**). Because $CD8^+$ T cells are the key players in the induction of cerebral malaria, we first evaluated the severity of symptoms at day 6 p.i. $CD160^{-/-}$ mice developed more severe cerebral malaria compared to WT mice (**Figure 5A**), although they exhibit a similar parasitemia (**Supplementary Figure 3**). Considering the similar number of brain-infiltrating $CD8^+$ T cells observed (**Figure 5B**), we further investigated their phenotypical profile. Only a fraction of $CD8^+$ T cells expressed CD160 in WT mice. However, due to the lack of a surrogate marker for $CD8^+CD160^+$ T cells, the isolation of this cell population in $CD160^{-/-}$ mice was not possible. Importantly, in the $CD160^{-/-}$ mice a deletion of 5 amino acids was introduced resulting in a premature stop codon (**Supplementary Figure 2**). However, the *Cd160* mRNA upstream of the deletion site is unchanged compared to the WT sequence. Therefore, we could detect *Cd160* mRNA both in WT and $CD160^{-/-}$ cells using a probe binding upstream of the deletion site (**Figure 5C**). We used *Cd160* mRNA probes for selective identification of *Cd160* $mRNA^+$ CD8 T cells in WT and $CD160^{-/-}$ mice. Although no differences in the frequency of GzmB or IFNγ expressing cells within the splenic $CD8^+$*Cd160*$mRNA^+$ T cells was observed, increased mean fluorescence intensity (MFI) for both molecules was detected in $CD160^{-/-}$ $CD8^+$ T cells compared to controls, thus confirming that CD160 controls $CD8^+$ T cell cytotoxic

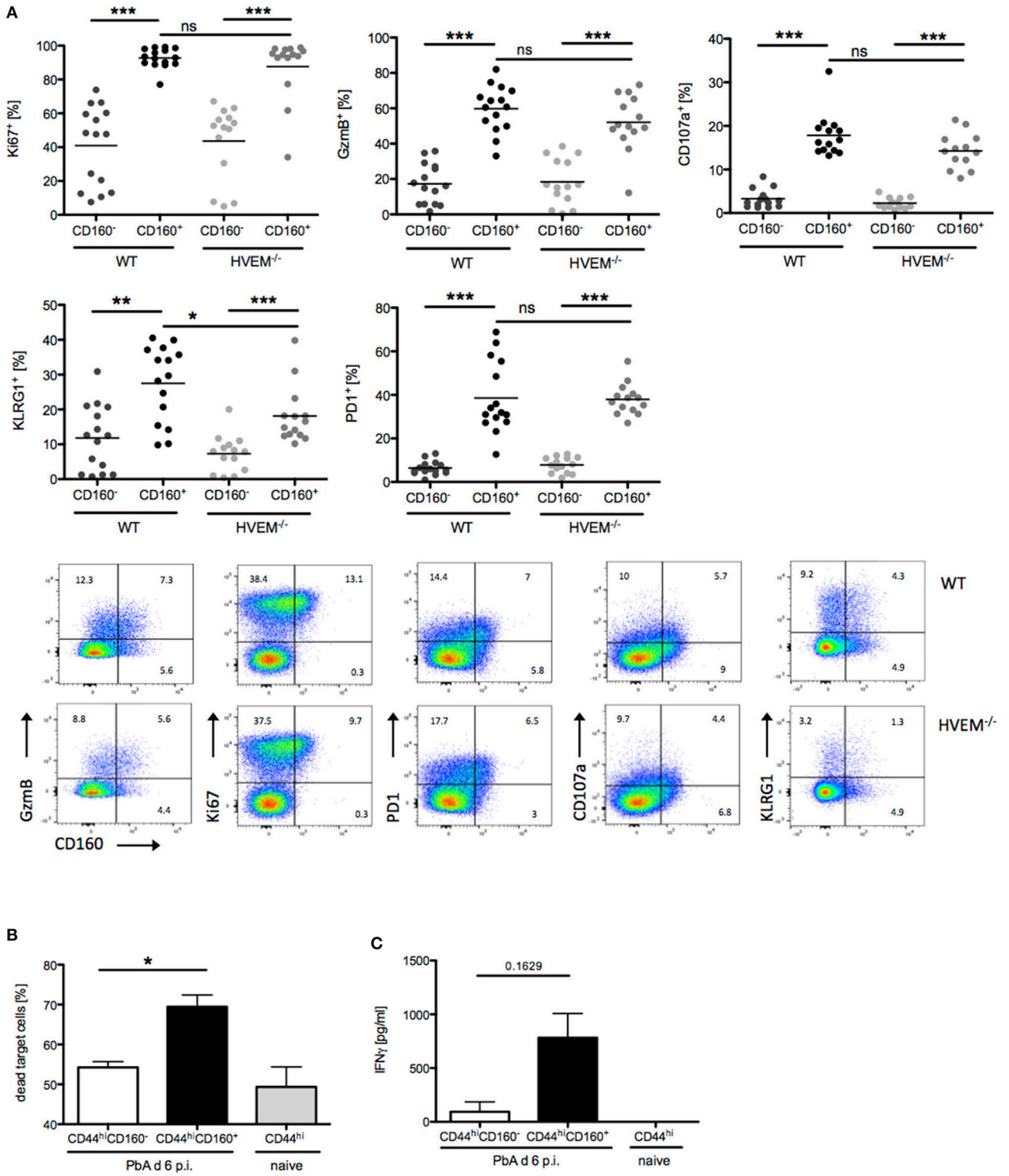

FIGURE 3 | CD160 characterizes highly activated and cytotoxic CD8$^+$ T cells during malaria. **(A)** Flow cytometric analysis of CD160$^{+/-}$CD8$^+$ T cells isolated from the spleen at d 6 p.i. Data were pooled from 3 independent experiments including 3–6 mice/group (total WT n = 14–15, HVEM$^{-/-}$ n=13–14). Representative plots are shown. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. **(B)** Flow cytometry based cytotoxicity assay performed on CD8$^+$CD44^{hi}CD160$^+$ and CD8$^+$CD44^{hi}CD160$^-$ effector cells FACS sorted from spleens of PbA infected mice at d 6 p.i., and co-cultured with naive splenocytes pulsed with Pb-specific peptides as target cells. Data were pooled from 4 independent experiments, in which a pool of splenocytes from 3–4 mice was used. This means ± SEM are shown. *$p = 0.0286$ **(C)** Release of IFNγ by effector cells, isolated as described in **(B)**, after 24 h co-culture with Pb-peptide pulsed Hepa1-6H cells. Pooled data of three independent experiments performed in at least triplicates is shown as means ± SEM.

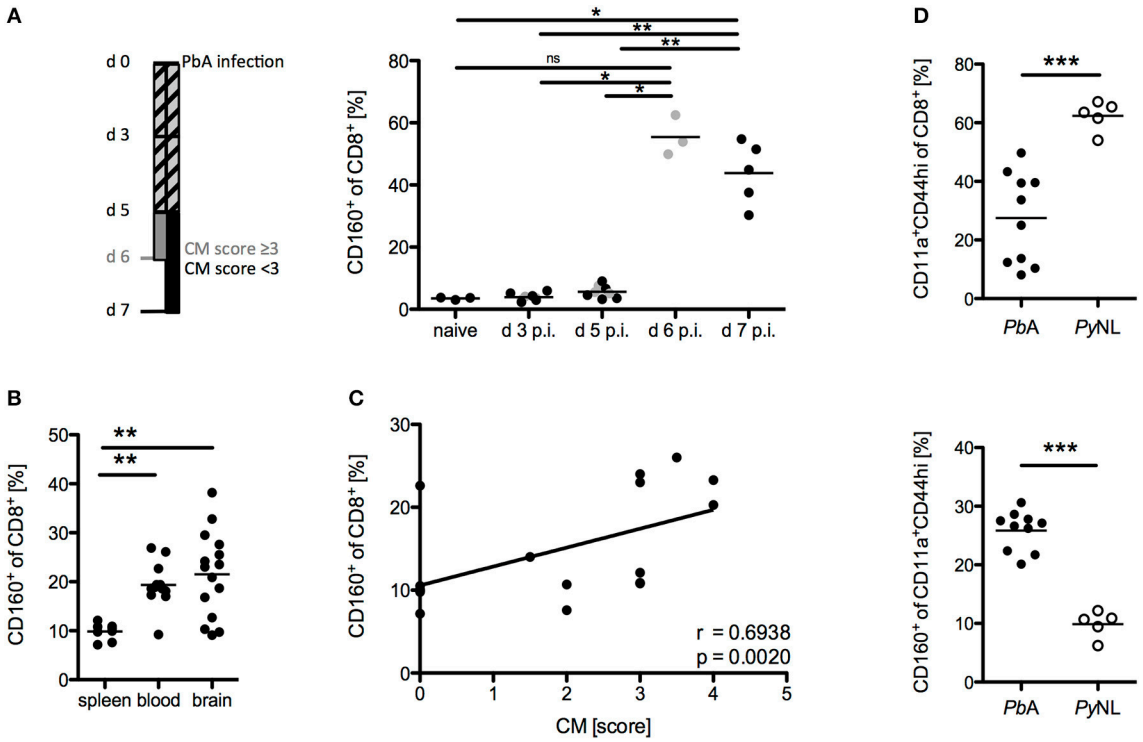

FIGURE 4 | $CD8^+CD160^+$ T cells are associated with cerebral pathology during PbA infection. **(A)** Flow cytometric analysis of the frequency of $CD8^+CD160^+$ T cells in the blood of WT mice, collected at the indicated days post PbA infection. Mice that developed severe cerebral malaria by d 6 p.i. (indicated in gray) were analyzed at day 6 whereas all other mice (indicated in black) were analyzed at d 7 p.i. Data were pooled from two independent experiments including up to 5 mice/group (naive $n = 3$, PbA $n = 8$) **(B)** Flow cytometric analysis of the frequency of $CD8^+CD160^+$ T cells in spleen, blood or brain of PbA infected WT mice. Data was pooled from 6 independent experiments including 2–4 mice/group (spleen $n = 8$, blood $n = 12$, brain $n = 15$) **(C)** Correlation of the severity of cerebral symptoms and frequency of splenic $CD8^+CD160^+$ T cells of PbA infected WT mice. Data were pooled from 4 independent experiments including 4–5 mice/group (total $n = 17$). **(D)** Frequency of activated ($CD11a^+CD44^{hi}$) $CD8^+$ T cells and $CD160^+$ T cells within the $CD11a^+CD44^{hi}$ cell population isolated from spleens of either PbA or PyNL infected mice. Data were pooled from two independent experiments including 2–5 mice/group (PbA $n = 10$, PyNL $n = 5$). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

function and IFNγ production. Of note, while CD160 did not regulate GzmB secretion, IFNγ production was still a CD160-dependent process in $CD8^+$ T cells in the brain (**Figure 5D**).

$CD8^+CD160^+$ T Cells Exhibit a Similar Phenotype in Human and Mice

We compared the T cell phenotypes in human peripheral blood of malaria patients and healthy controls (HC) in light of the previous findings in the mouse model. Unfortunately, tools for the staining of *Plasmodium*-specific $CD8^+$ T cells have not been developed yet. Additionally, high background level of $CD8^+$ T cells activated by e.g., previous or chronic viral infection might interfere with recent *Plasmodium*-specific $CD8^+$ T cell activation. For this reason, we investigated the presence of $CD8^+$ T cell biomarkers specifically induced during malaria. As additional controls, we used peripheral blood samples from patients suffering from autoimmune liver disease [autoimmune hepatitis (AIH), primary billary cholangitis (PBC), primary sclerosing cholangitis (PSC)] or chronic hepatitis B virus (HBV) infection. $CD8^+$ T cells were stained for activation (CD28), proliferation (Ki67), cytotoxicity (GzmB, Perforin) and differentiation markers (PD-1). We found that $CD8^+CD28^+GzmB^+$ T cells were enriched in malaria patients compared to healthy controls and patients suffering from AIH, PBC, PSC or chronic HBV infection (**Figure 6A**). Indeed, CD160 was co-expressed with GzmB and CD28, suggesting that it is induced during acute *Plasmodium* infection in $CD8^+$ T cells (**Figure 6A**). Subsequently, we analyzed the expression of the cytotoxic molecule GzmB and Perforin the proliferation marker Ki67 and the co-inhibitory receptor PD-1, both in $CD8^+CD160^+$ and $CD8^+CD160^-$ T cells from healthy controls or malaria patients. While no difference in Ki-67 expression was observed, the frequency of cytotoxic ($GzmB^+$ and $Perforin^+$) and terminally differentiated ($PD\text{-}1^+$) cells was increased in $CD8^+CD160^+$ T cells compared to the control counterpart (**Figure 6B**).

Taken together, our results show that CD160 expression identifies a population of highly activated and cytotoxic $CD8^+$ T cells in peripheral blood. Of note, this feature is conserved in murine and human $CD8^+$ T cells.

DISCUSSION

A tight control of $CD8^+$ T effector cell function is crucial in order to allow efficient clearance of invading pathogens without the development of immunopathology due to an

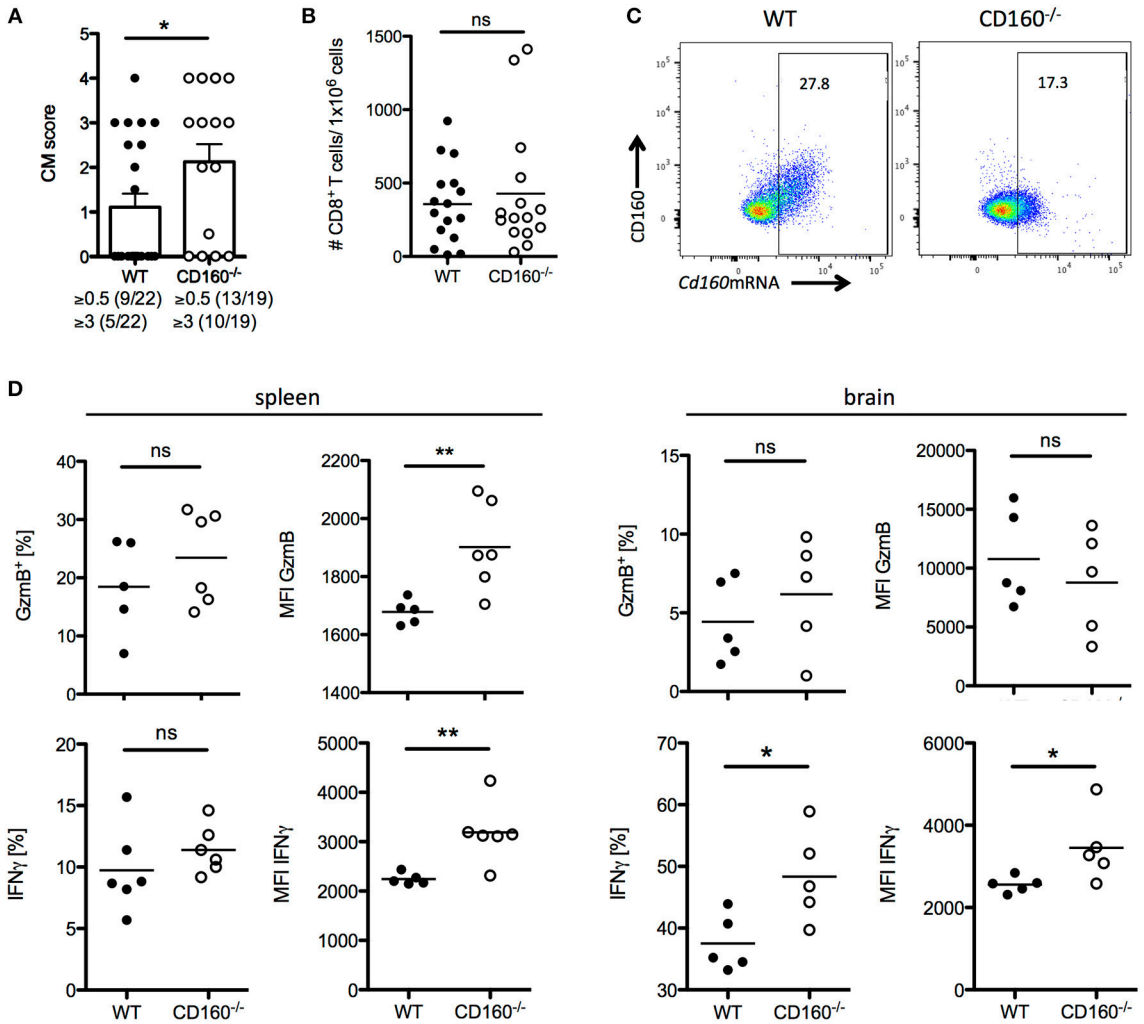

FIGURE 5 | CD160 restricts T cell mediated cerebral pathology. **(A)** Severity of cerebral symptoms was monitored in WT and CD160$^{-/-}$ mice at d 6 post PbA infection. Data were pooled from 4 independent experiments including 3–9 mice/group. **(B)** Absolute number of brain-infiltrating CD8^{+} T cells in PbA infected WT and CD160$^{-/-}$ mice was measured by flow cytometry. Data were pooled from 3 independent experiments including 3–8 mice/group (WT $n = 16$, CD160$^{-/-}$ $n = 15$). **(C)** Representative dot plots of CD8^{+} T cells from the spleen from PbA infected mice stained for CD160 protein and *Cd160* mRNA. **(D)** CD8^{+}*Cd160* mRNA^{+} T cells from WT or CD160$^{-/-}$ mice were analyzed for frequency (%) and mean fluorescence intensity (MFI) of granzyme B (protein) and IFNγ (mRNA). Representative data from one out of two experiments is shown. *$p < 0.05$ and **$p < 0.01$.

overwhelming response. Considering that co-inhibitory and co-stimulatory receptors are important regulators of CD8^{+} T effector function, we aimed to better understand how the HVEM-CD160 axis shapes the CD8^{+} T cell response during malaria.

HVEM is broadly expressed in CD4^{+} and CD8^{+} T cells (15). In this study we focused on CD8^{+} T cells since they are central for mediating cerebral pathology. By taking advantage of adoptive transfer experiments of control and HVEM-deficient CD8^{+} T cells in the same WT recipient, we can address the intrinsic and direct contribution of HVEM on CD8^{+} T cell function. In view of the equal proliferation observed in CD8^{+} T cells from WT and HVEM$^{-/-}$ mice in *in vivo* and *in vitro* experimental settings, we conclude that HVEM seems to provide pro-survival signals, which are essential for the persistence of CD8^{+} T effector cells. Consequently, the number and frequency of terminally differentiated effector cells is reduced. However, CD8^{+} T cells lacking HVEM are not impaired in their capacity to express e.g., the effector molecule GzmB. Our data on the role of HVEM in the control of CD8^{+} T cell persistence are supported by studies showing the pro-survival function of HVEM in memory T cells performed in the context of listeria, influenza and vaccinia virus infection or with the model antigen OVA (19–22). Our data suggest that the reduced frequency of highly activated CD8^{+} T cells, which can be identified by the expression of CD160, results in the reduced severity of ECM.

The HVEM-ligand CD160 has been reported to be expressed by cytotoxic and IFNγ producing NK and CD8^{+} T cells (31,

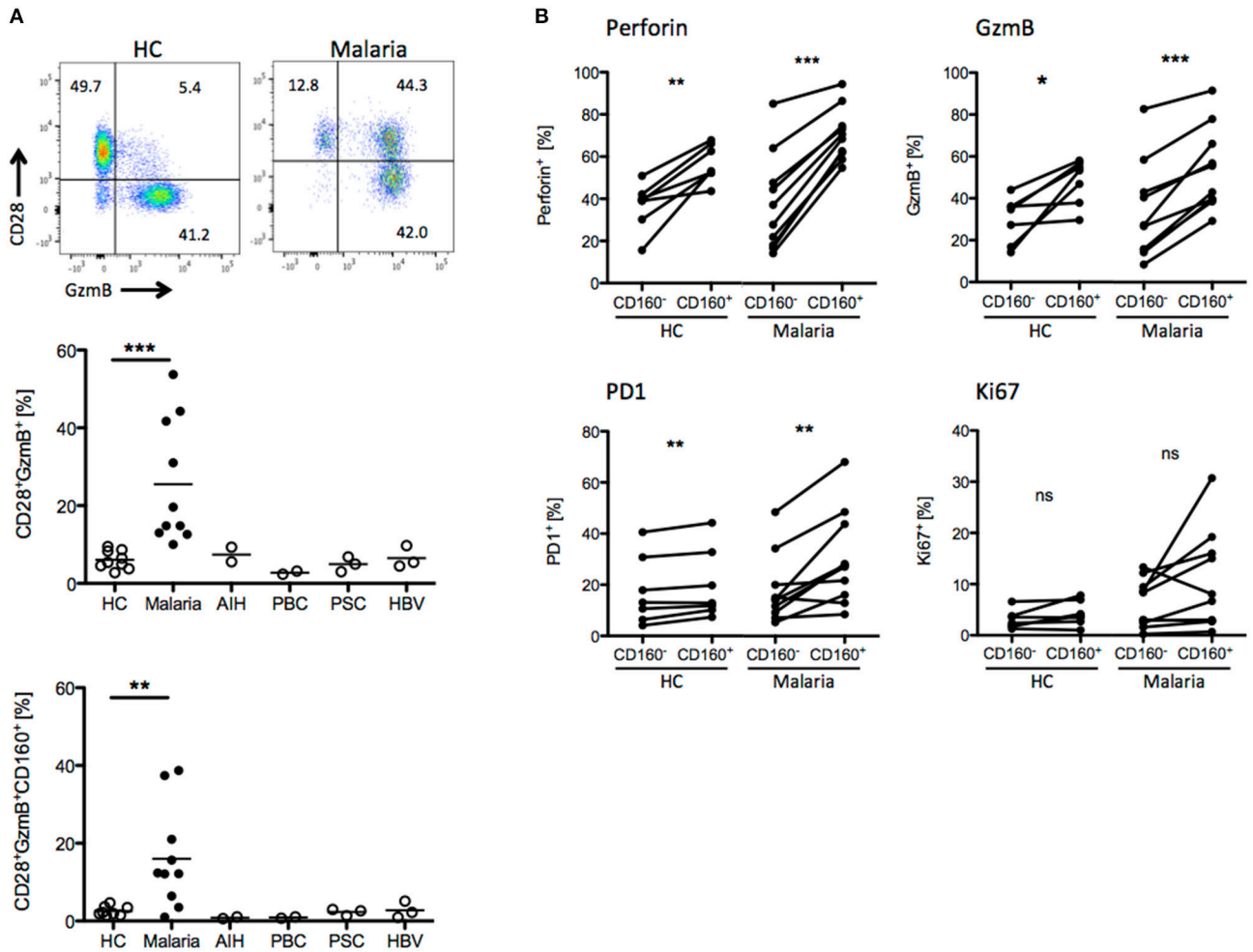

FIGURE 6 | $CD8^+CD160^+$ T cells exhibit a similar phenotype in human and mice. **(A)** Whole peripheral blood samples from healthy controls (HC, $n = 9$), Malaria patients (Malaria, $n = 10$) and patients suffering from autoimmune hepatitis (AIH, $n = 2$), primary biliary cholangitis (PBC, $n = 2$), primary sclerosing cholangitis (PSC, $n = 3$) and chronic hepatitis B virus infection (HBV, $n = 3$) were analyzed by flow cytometry for the expression of CD28, GzmB, and CD160 on $CD8^+$ T cells. Representative plots are shown. **(B)** Expression of Perforin, GzmB, PD1, and Ki67 in $CD8^+CD160^+$ and $CD8^+CD160^-$ T cells isolated from healthy controls ($n = 7$) or malaria patients ($n = 10$) was analyzed by flow cytometry. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

32, 44). Based on this, we analyzed its function in $CD8^+$ T cells during ECM. CD160 competes with the co-inhibitory receptor BTLA for the binding to HVEM. We have previously shown that targeting BTLA with an agonistic antibody can restrict cerebral pathology (7). However, BTLA is expressed by a variety of cell types including innate and adaptive immune cells. Consequently, BTLA not only restricts the T cell mediated immunopathology during cerebral malaria but also the protective immunity against a non-lethal infection with *P. yoelii* by the suppression of phagocytes and B cells (8). In contrast to BTLA, our data shows that CD160 expression is restricted to highly activated $CD8^+$ T cells.

The cytotoxic phenotype of $CD8^+$ T cells, which express CD160 has already been described by others (44). Only one study has indeed analyzed the direct function of CD160 in NK cells by using a $CD160^{-/-}$ mouse model. In this scenario, CD160 deficient NK cells showed in melanoma and RMA-S lymphoma tumor models a defect in tumor control due to impaired IFNγ production (41). Our results regarding $CD8^+$ T cells hint into a different direction. First, $CD160^{-/-}$ mice develop more severe cerebral pathology suggesting a co-inhibitory rather than a co-stimulatory function of CD160. Second, we observed an increase in IFNγ mRNA in $CD8^+$ T cells of $CD160^{-/-}$ mice. The role of CD160 might be different in NK and $CD8^+$ T cells because of a different composition of co-receptors expressed by the respective cell types. In T cells, engagement of CD160 leads to association to the CD3ζ chain (29, 30). In contrast, in NK cells CD160 has been described to co-localize with CD2 (45). Furthermore, the CD160-ligand might also influence the effect of CD160 engagement. Besides HVEM, CD160 binds to classical and non-classical MHCI molecules (27, 31, 32, 46). A dual function of co-receptors depending on e.g. the ligand or the cell type they

are expressed by has already been described for other receptors such as 2B4 and Tim-3 (47, 48).

It might be possible that the lack of CD160 disturbs the balanced signaling between co-stimulatory HVEM and co-inhibitory BTLA. However, this is unlikely due to the high expression levels of BTLA and HVEM compared to the low level of CD160 during ECM. But even if HVEM signaling is impaired by the loss of CD160, this should lead to reduced immunopathology according to our data obtained in HVEM$^{-/-}$ mice. However, we see an aggravated pathology in CD160$^{-/-}$ mice. Hence we conclude, that the phenotype is directly mediated by the loss of CD160 rather than by influencing the HVEM-BTLA axis.

In order to analyse the function of CD160, besides its genetic ablation, several studies utilized soluble CD160 molecules or antibodies to disrupt the interaction of CD160 with its respective ligand in different experimental settings. In a model of cardiac allograft transplantation, treatment with CD160Ig was beneficial to control CD8$^+$ mediated allograft rejection (42). The authors propose that in this context CD160 acts as a co-stimulatory molecule on CD8$^+$ T cells and thus the use of CD160Ig reduces the amount of secreted IFNγ by CD8$^+$ T cells (42). Similarly, CD160Ig treatment enhances the graft survival in a model of skin allograft rejection when treatment is combined with anti-CD40L antibody (43). Of note, when treated with soluble CD160 not only is the CD160-HVEM interaction affected due to their shared binding region, but so is the binding of BTLA to HVEM. Furthermore, it is not clear whether the soluble CD160 preferentially binds to either HVEM or MHCI or both but the higher binding affinity of CD160 for HVEM suggest that it binds to HVEM. In this case, the signaling of CD160 expressed by CD8$^+$ T cells might not be completely blocked but instead interaction with MHCI is favored. Additionally, in mice infected with *Citrobacter rodentium*, CD160 expressed by intraepithelial lymphocytes triggers HVEM on epithelial cells and induces an anti-microbial response in conjunction with IL-22 (28). Finally, *in vitro* cross-linking of CD160 is reported to either stimulate or inhibit the expressing cells, depending on co-stimulation and cell type (15, 27, 29–31, 49–51). In summary, most studies suggest a co-stimulatory role of CD160. However, it is important to consider that the antibody could have an agonistic rather than a blocking function. Because of these limitations we decided to generate a CD160-deficient mouse.

In contrast to their absence in naïve mice, we detect CD8$^+$CD160$^+$ T cells in peripheral blood of healthy donors. Other groups have already described CD160 expression in CMV, EBV and HIV-specific CD8$^+$ T cells in chronically infected patients (49, 52, 53). These data are in line with our results, which show a constant expression of CD160 in CD8$^+$ T cells from mice infected with PyNL even after natural clearance of the parasite. Taken together, these data suggest that once CD160 is induced on murine and human CD8$^+$ T cells, its expression is maintained and that this expression is associated with increased levels of CD8$^+$ T cell activation and cytotoxicity.

CD160 has been selected as a candidate target for the treatment of vascular-eye diseases. Furthermore, it has also been considered for therapeutic intervention in cancer patients, according to the anti-angiogenic effect of CD160 antibodies on growing endothelial cells (54, 55). Based on our data, we propose that CD160 delineates highly activated CD8$^+$ T cells and might be useful to restrict immunopathology in the future.

In conclusion, in this study we have shown that the HVEM-CD160 axis is critical in the fine-tune regulation of stimulatory and inhibitory signals in CD8$^+$ T cells during blood-stage malaria.

AUTHOR CONTRIBUTIONS

FM and TJ designed the experiments. FM and NS conducted the experiments. FM, NS, TJ, and LB analyzed and interpreted the data. JS and CS recruited patients and contributed samples. FM, TJ, and LB wrote the manuscript and prepared the figures. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02611/full#supplementary-material

Supplementary Figure 1 | CD160 remains expressed by CD8$^+$ T cells, while the proliferation marker Ki67 is reduced to steady-state conditions by d 20 p.i. Lymphocytes from peripheral blood from PbA infected mice were isolated at indicated time points and CD8$^+$ T cells were analyzed by flow cytometry for CD160 and Ki67 expression. One of two independent experiments including 3 mice/group is shown.

Supplementary Figure 2 | Characterization of CD160$^{-/-}$ mice. **(A)** CD160 amino acid sequence, disulfide bounds, glycosylation sites, the lipidation site and known structural domains are depicted according to uniprot data. For the -/- protein, the amino acid sequence subsequent of the site of deletion was calculated by the ExPASy translation tool. **(B)** Ear tissue lysates were used for amplification of the DNA sequence including the CRISPR/Cas9 target site by PCR. The size of the PCR products was analyzed by agarose gel electrophoresis. An exemplary gel with samples from eight mice (M1-M8, lane 3-10) and a negative control without template (H_2O, lane 2) is shown. The PCR product size is annotated according to the 500 bp ladder (lane 1). **(B)** PCR products were digested by Bpu10I and the size again analyzed by agarose gel electrophoresis. The genotype referring to the analyzed mice is annotated: +/+ wild type, +/- heterozygous, -/- homozygous knockout. (C,D) WT and CD160$^{-/-}$ mice were infected with PbA and organs were collected at d 6 p.i. CD3$^+$ cells from the spleen **(C)** or blood **(D)** were analyzed by flow cytometry for CD160 expression. Representative plots of two independent experiments are shown. **(E)** Intestinal intraepithelial cells from naïve WT and CD160$^{-/-}$ mice were analyzed by flow cytometry for CD160 expression on non-hematopoietic cells (CD8$^-$CD45$^-$) and hematopoietic cells (CD45$^+$), being positive or negative for CD8. Representative

plots of two independent experiments are shown. Frequency of T cell subsets (CD4/CD8; TCRβ/γδ), B cells (CD19) and NK cells (NK1.1) within splenocytes **(F)** and CD4/CD8 T cells in the thymus **(G)** was assessed by flow cytometry. Representative plots out of two independent experiments are shown.

Supplementary Figure 3 | Parasitemia of HVEM$^{-/-}$ and CD160$^{-/-}$ mice. The frequency of PbA infected RBC at day 6 p.i. of HVEM$^{-/-}$ **(A)** or CD160$^{-/-}$ **(B)** mice is shown. Data is pooled from 8 **(A)** or three **(B)** independent experiments including 3–6 mice/group. $*p < 0.05$.

Supplementary Figure 4 | Gating strategy for murine cells. Flow cytometry data of murine samples was gated according to the strategy shown.

Supplementary Figure 5 | Gating strategy for human cells. Flow cytometry data of human samples was gated according to the strategy shown.

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Infection-Induced Resistance to Experimental Cerebral Malaria Is Dependent Upon Secreted Antibody-Mediated Inhibition of Pathogenic CD8$^+$ T Cell Responses

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Cerebral malaria (CM) is one of the most severe complications of *Plasmodium falciparum* infection. There is evidence that repeated parasite exposure promotes resistance against CM. However, the immunological basis of this infection-induced resistance remains poorly understood. Here, utilizing the *Plasmodium berghei* ANKA (PbA) model of experimental cerebral malaria (ECM), we show that three rounds of infection and drug-cure protects against the development of ECM during a subsequent fourth (4X) infection. Exposure-induced resistance was associated with specific suppression of CD8$^+$ T cell activation and CTL-related pathways, which corresponded with the development of heterogeneous atypical B cell populations as well as the gradual infection-induced generation and maintenance of high levels of anti-parasite IgG. Mechanistically, transfer of high-titer anti-parasite IgG did not protect 1X infected mice against ECM and depletion of atypical and regulatory B cells during 4X infection failed to abrogate infection-induced resistance to ECM. However, IgMi mice that were unable to produce secreted antibody, or undergo class switching, during the repeated rounds of infection failed to develop resistance against ECM. The failure of infection-induced protection in IgMi mice was associated with impaired development of atypical B cell populations and the inability to suppress pathogenic CD8$^+$ T cell responses. Our results, therefore, suggest the importance of anti-parasite antibody responses, gradually acquired, and maintained through repeated *Plasmodium* infections, for modulating the B cell compartment and eventually suppressing memory CD8$^+$ T cell reactivation to establish infection-induced resistance to ECM.

Keywords: cerebral malaria, T cells, B cells, spleen, brain, antibody

INTRODUCTION

Malaria remains one of the most prevalent and severe diseases in the world, responsible for 445,000 deaths, principally in Sub Saharan Africa, in 2016 (1). The majority of malarial morbidity and mortality are attributable to a small number of distinct but frequently overlapping complications, of which, cerebral malaria is one of the most severe (2, 3). In endemic regions, children under 5 years of age are disproportionally susceptible to cerebral malaria whereas older children and adults, despite often harboring very high parasite burdens, rarely develop severe disease (4).

It is believed that the age-associated protection from cerebral malaria in endemic regions is driven by repeated parasite-exposure, and resultant reprogramming of anti-*Plasmodium* immunity, rather than natural evolution of immune components in the maturing host immune system (5–9). Indeed, adults remain susceptible to severe malarial disease in non-endemic malarial areas and in regions of unstable transmission (4). Nevertheless, the precise number of infectious episodes necessary to provide immunity to severe malaria has yet to be definitively defined (10). Moreover, despite substantial research, the nature, and identity of the immune responses that develop following natural repeated exposure to prevent cerebral malaria are also poorly understood (5–9).

Anti-parasite antibodies, $Foxp3^+$ regulatory T cells, IL-10 secreting T cells, and tolerance to malaria toxins, such as GPI and hemozoin, have all been postulated to play important roles in the establishment of immune balance during malaria; however, how these responses develop and their relative contribution to infection-induced protection against severe disease, is mostly unknown (5–9, 11, 12). There is increasing evidence that atypical B cell populations characterized by expression of various markers including CD11c and T-bet develop in response to chronic and repetitious *Plasmodium* exposure (13, 14). Nevertheless, the function of atypical B cells and their influence in regulating resistance to severe malarial complications remain unclear (13–19). Indeed, whilst atypical memory B cells have been shown to be superior active sources of anti-parasite antibody than traditional memory B cells (15), in other studies they have been shown to exhibit restricted activation and impaired capacity to differentiate into antibody or cytokine producing cells following re-stimulation (13). Atypical memory B also express high levels of regulatory receptors and may exert important immunoregulatory functions in suppressing inflammation during malaria, contributing to anti-disease immunity (14, 17–19).

In this study we have utilized the *Plasmodium berghei* ANKA (PbA) murine model of experimental cerebral malaria (ECM) to examine the immunological basis of exposure-induced resistance against malarial cerebral pathology. To date, the ECM model (20) has been significantly underused in the study of immune mechanisms that provide infection-induced protection against severe malaria disease. Indeed, the physiological immune pathways developed following repeated PbA infection that prevent ECM remain unknown. Here we demonstrate that three rounds of infection-drug cure are required to promote robust resistance to ECM during subsequent fourth (4X) infection. We show that infection-induced resistance to ECM is associated with the significant expansion of atypical B cell populations and repression of memory $CD8^+$ T cell reactivation, and that this protection is abrogated in mice unable to produce secreted antibody, or undergo class switching. Protection, however, cannot be recapitulated in primary infected mice following passive transfer of plasma from resistant repeatedly infected mice. Moreover, protection is intact in repeatedly infected mice in which atypical and regulatory B cells are acutely depleted. The results in this study provide new evidence for the importance of antibody in mediating protection against cerebral malaria and suggest antibody is required throughout and post-repeated infections to orchestrate gradual modulations in immune responses that ultimately underpin protection.

MATERIALS AND METHODS

Mice and Infections

6–8 week old C57BL/6 mice were purchased from Charles River. IgMi mice (21, 22) and littermate WT mice were bred at the University of Manchester. All mice were maintained in individually ventilated cages at the University of Manchester. BALB/c mice were used for production of *P. berghei* parasite lysate at the University of Oxford. Cryopreserved GFP-expressing *P. berghei* ANKA parasites of clone 15cy1 (23) were thawed and passaged through mice before being used to infect experimental mice via intravenous (i.v.) injection of 1×10^4 parasitized red blood cells (pRBCs) in the tail vein. Infected mice were monitored for neurological symptoms (paralysis, ataxia, convulsions, and coma occurring between day 6 and 10 post-infection). Parasitemia was measured from day 3 post-infection (p.i.) by examination of Giemsa-stained thin blood. Drug cure was achieved on specified days by six, daily, intraperitoneal (i.p.) injections of 30 mg/kg chloroquine combined with 30 mg/kg artesunate in PBS. In some experiments mice were administered on indicated days of 4X infection, via intraperitoneal (i.p.) injection, 300 μg anti-IL-10R (1B1.3A), 250 μg anti-CD20mAb (5D2), or 250 μg anti-Ragweed (control) mAb (Abs from Bioxcell or Genentech, Inc.). In some experiments mice were administered on indicated days of 1X infection, via i.p. injection, 500 μl of heat inactivated plasma (heated at 56°C for 30 min to destroy cytokines and complement), obtained from 4X infected mice (day 7/8 of infection) or from age matched naïve mice. ECM development was assessed using a well-established grading system (24): 1=no signs; 2=ruffled fur/and or abnormal posture; 3=lethargy; 4=reduced responsiveness to stimulation and/or ataxia and/or respiratory distress/hyperventilation; 5= prostration and/or paralysis and/or convulsions. Stages 2–3 were classified as prodromal signs of ECM and stages 4–5 were classified as late stage ECM. All animals were euthanized by rising concentration of CO_2 when observed at stage 4 or 5.

RNA Isolation

Spleen sections were isolated from mice prior to whole-body perfusion of PBS. Brains were isolated from mice following intracardial whole-body perfusion of PBS. Tissue was snap

frozen in liquid nitrogen and stored at −80°C until use. RNA isolation from spleen sections and whole brains was performed by homogenizing brains in Trizol and using lipid tissue RNA easy kits according to the manufacturer's instructions (RNeasy Lipid Tissue Mini Kit, Qiagen). Isolated RNA was DNase treated to remove genomic DNA prior to QC analysis and use in microarray or NanoString analysis.

Microarray and Gene Expression Analysis

The global gene expression profiles of brains from uninfected, 1X infected, post-3X infected mice and 4X infected mice were probed using the Affymetrix GeneChip® Mouse Genome 430 2.0 microarray containing 34,000 genes. Technical quality control and outlier analysis was performed with dChip (V2005) [www.dchip.org: (25)] using the default settings. Background correction, quantile normalization, and gene expression analysis were performed using RMA in Bioconductor (26). To establish relationships and compare variability between samples, principal components analysis (PCA) was used as this method reduces the effective dimensionality of complex gene-expression space without significant loss of information (27). PCA was performed with Partek Genomics Solution (version 6.5, Copyright 2010, Partek Inc., St. Charles, MO, USA). Differential expression analysis was performed using Limma using the functions lmFit and eBayes (28). 1,957 probe-sets were identified by pairwise comparisons (fold change $<$ or $>$ 1.5 and qvalue $<$ 0.05) between 1X infected vs. uninfected and/or 4X infected vs. aged uninfected groups. The expression level of each probeset was normalized to the naïve average (in log scale, the naïve average expression was calculated and subtracted from each expression level), and then the standard deviation was normalized to 1 (expression level was divided by the standard deviation). The differentially expressed probesets were ranked by clustering the mean expression levels in each group (expression in each group normalized by z-transformation of the mean in log scale), by k-means clustering into 5 clusters, and then ranked by hierarchical clustering. Gene ontology analysis was performed using DAVID Functional Annotation Bioinformatics Microarray Analysis database.

Nanostring Analysis

The nCounter Gene Expression assay (Nanostring Technologies, Seattle, USA) was performed according to the manufacturer's instructions. Transcript counts were normalized to the relevant housekeeping genes using the nSolver Analysis Software (vers. 2.5; Nanostring Technologies).

Flow Cytometry

Spleens were mashed and cell suspensions were generated by homogenizing tissue through a 70 μm cell sieve (BD Biosciences) and subjected to RBC lysis (BD Bioscience). Brains were finely minced and leukocytes isolated using the single-step percoll method (29). Cell pellets were subjected to a RBC lysis step. Absolute cell numbers were determined by microscopy using a haemocytometer and live/dead differentiation was performed using the trypan blue exclusion cell viability assay (Sigma). Isolated leukocytes were surface stained with anti-mouse CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), CD11c (N418), CD19 (6D5 or 1D3), CD45 (30-F11), CD62L (MEL-14), CD80 (16-10A1), CD138 (281-2), ICOS (15F9 or C398.4A), KLRG1 (2F1), IgD (11-26c.2a), IgM (RMM-1), MHC II (M5/114.15.2), PDCA-1 (927), and PDL-1 (MIH5 or 10F.9G2). Intracellular staining for Foxp3 (FJK-16s), granzyme B (GB11), IFNγ (XMG1.2), Ki67 (SolA15) and Tbet (4B10) was performed, after treatment with Foxp3 fixation/permeabilisation buffer (eBioscience). IFNγ staining was performed following 4 h *in vitro* stimulation of splenocytes with 50 ng/mL PMA / 2.5 ug/mL ionomycin. Dead cells were excluded using LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Life Technologies). Fluorescence minus one (FMO) controls were used to set gates. Cells were analyzed with a BD LSR II or Fortessa (Becton Dickinson) using BD FACSDiva software (Becton Dickinson). Data were analyzed with FlowJo (Tree Star Inc.). All antibodies were from eBioscience and Biolegend.

Histology

Brains were processed and stained with Haematoxylin/Eosin (H & E) as described (17). Briefly, brains were isolated from mice perfused with PBS followed by 4% paraformaldehyde (pfa). Brains were stored in 20% sucrose/4% pfa for 24 h at (4°C) before being transferred to 20% sucrose/PBS for a further 24 h (4°C). Coronal sections were cut using a sledge-microtome at a thickness of 30 μm. Sections were mounted and stained with Haematoxylin/Eosin.

Immunofluorescence

Brain sections were stained with anti-GFP (A-21311, Life Technologies) and anti-CD31 (MEC 13.3, BD Pharmingen) as described (20). Sections were counterstained in DAPI (Sigma-Aldrich) then cover-slipped in ProLong Diamond anti-fade Mountant (Life Technologies).

ELISA

Serum anti-*P. berghei* merzoite surface protein 1 C-terminal 19 kDa region (*Pb*MSP1$_{19}$) and anti-*P.berghei* ANKA antibody endpoint titers were determined as previously described (30), Briefly, 96 well ELISA plates (Thermo Scientific) were coated with *Pb*MSP1$_{19}$-glutathione S-transferase (GST) fusion protein [0.5 μg/ml] or *P.berghei* ANKA blood-stage parasite lysate, generated as previously described (31) [0.5 μg/ml], respectively, before being incubated overnight at 4°C, blocked with PBS with 1% BSA at 37°C for 1 h, and diluted mouse sera added in duplicate wells. Plates were incubated at 37°C for 2 h before antibodies were detected using alkaline phosphatase conjugated goat anti-mouse total IgG (Sigma-Aldrich) (1:3,000) for 1 h at 37°C and developed with p-nitrophenylphosphate (Sigma-Aldrich) with absorbance readings at taken at OD_{405nm}. Anti-GST responses were obtained by running concurrent wells coated with GST protein [0.29 μg/ml] and subtracted from the respective *Pb*MSP1-GST OD_{405nm} readings. Endpoint titers were determined as the calculated dilution at which the OD_{405nm} equaled no sera control wells for anti-parasite, and zero for the GST subtracted anti-*Pb*MSP1$_{19}$ ELISA.

Quantification of Plasma Cytokine Levels

The concentrations of IL-2, IFN-γ, TNF, and IL-10 in plasma were measured using a ProcartaPlex Mouse Cytokine & Chemokine Panel (26 plex) (eBioscience) on a Luminex® 100/200™ System, following the manufacturer's instructions.

Statistical Analysis

All statistical analyses were performed using GraphPad PRISM (GraphPad Software, USA). Data were tested for normality using the Shapiro-Wilk normality test. For normally distributed data, comparisons between two groups were made using a Student's *t*-test, with Welch's correction and between multiple groups using a one-way ANOVA with Tukey's test for multiple comparisons. For non-parametric data, comparisons between two groups were made using a Mann-Whitney test and between multiple groups using a Kruskal-Wallis with Dunn's test for multiple comparisons. N-numbers in figure legends refer to the number of biological replicates used to generate the data shown in the figure.

RESULTS

Repeated Cycles of Infection and Drug-Cure Protects Mice Against ECM

To recapitulate the natural repeated *Plasmodium falciparum* infections experienced by humans in endemic regions (10) we adapted the established PbA model of ECM (**Figure 1**). C57BL/6 mice were infected with PbA and treated with anti-malarial drugs (artesunate, the front line treatment for severe malaria and chloroquine, as a representative quinolone-containing drug) prior to the development of fulminant ECM (on day 5 or 6, depending upon infection cycle) (experimental schematic in **Figure 1A)**. This cycle of infection-drug cure was repeated up to three times, with a minimum interval of 30 days between cessation of drug treatment and reinfection. Mice gradually acquired resistance to ECM, with mice experiencing a fourth infection (4X infected) being highly resistant to cerebral complications, which typically developed between 6 and 8 days post-infection **(Figure 1B)**. Repeatedly infected mice also gradually acquired a degree of parasite control as 4X infected mice exhibited reduced parasite burdens compared with other infected groups during the early phase of infection (**Figure 1C**); however, many of the mice subsequently developed transient high level peripheral parasite burdens, which were cleared in 80% of mice by day 30 of infection (**Figure S1A** and **Supplementary Table 1)**. As expected, very few histopathological features of ECM [as defined in [**20**]], were observed in qualitative analyses of brains from 4X infected mice, in contrast to evidence of pRBC accumulation (left panel), hemorrhage (middle panel) and blocked vessels (right panel) observed in brains from 1X infected mice **(Figure 1D** and **Figure S1B)**.

This model, therefore, mimics the gradual, infection-induced resistance to cerebral malaria seen in humans, in which resistance to cerebral pathology corresponds with reduced parasite accumulation in the brain and partial, albeit incomplete, control of patent peripheral parasitaemia.

Repeated Parasite Exposure Regulates Intracerebral CD8$^+$ T Cell Activity

Brain migrating CD8$^+$ T cells play a major role in the pathogenesis of ECM (32). Thus, we investigated whether infection-induced resistance to ECM was associated with the attenuation of intracerebral CD8$^+$ T cell responses. As anticipated, the numbers of CD8$^+$ T cells were reduced in the brains of 4X infected mice (day 8: no ECM) compared within brains of 1X infected mice (day 8: late stage ECM) (**Figures 2A,B**). Moreover, CD8$^+$ T cells displayed a less activated phenotype in the brains of 4X infected mice compared with 1X infected mice, as evidenced by significantly lower expression of granzyme B (**Figures 2C,D**).

To assess the potential intracerebral events that led to reduced CD8$^+$ T cell recruitment and CTL functionality in 4X infected mice, we contrasted the transcriptome of whole brains from 1X infected mice, from 4X infected mice, and from uninfected mice. Brains from the three different groups (from day 8 of both 1X and 4X infection) exhibited distinct transcriptional signatures (**Figures 2E,F**). Genes differentially expressed in brains of 1X infected or 4X infected mice compared with brains from uninfected mice clustered into 5 distinct clusters, with various different biological pathways relating to immune system activation and function being enriched in each cluster (**Figure S2**, **Supplementary Table 2)**.

Filtering the dataset in **Figure S2** (i.e., the genes differentially expressed in brains of 1X or 4X infected mice compared with naïve mice) further, we identified the genes that were also differentially expressed specifically in brains of 4X compared with 1X infected mice (1402 genes in total: **Supplementary Table 3**). These filtered genes were significantly enriched within immunological processes that included defense response, regulation of apoptosis, chemotaxis, CTL activity, antigen processing and presentation, and cell adhesion (selected genes in the biological processes are presented in **Figure 2G** and full pathways are presented in **Figure S2**). In general, the majority of the pro-inflammatory genes (including IL-6 and IFN-γ gene networks) were expressed at higher levels in brains from 1X infected compared with 4X infected mice (**Figure 2G** and **Figures S2**, The differences in expression of key genes involved in antigen processing and presentation, chemotaxis and CTL activity was verified by NanoString analysis (**Figure S3**). Importantly, the brain transcriptome in pre-4X infected mice (i.e., mice that had undergone three rounds of PbA infection and drug cure but did not receive a further infection) was almost identical to that observed in uninfected mice (**Figures 2H,I**).

Combined, these results show that exposure-induced resistance to ECM is associated with both the reduction in CD8$^+$ T cell accumulation and CD8$^+$ T cell cytolytic activity within the brain. Moreover, transcriptional differences in the brain are only apparent during active infection, and are not maintained between infections.

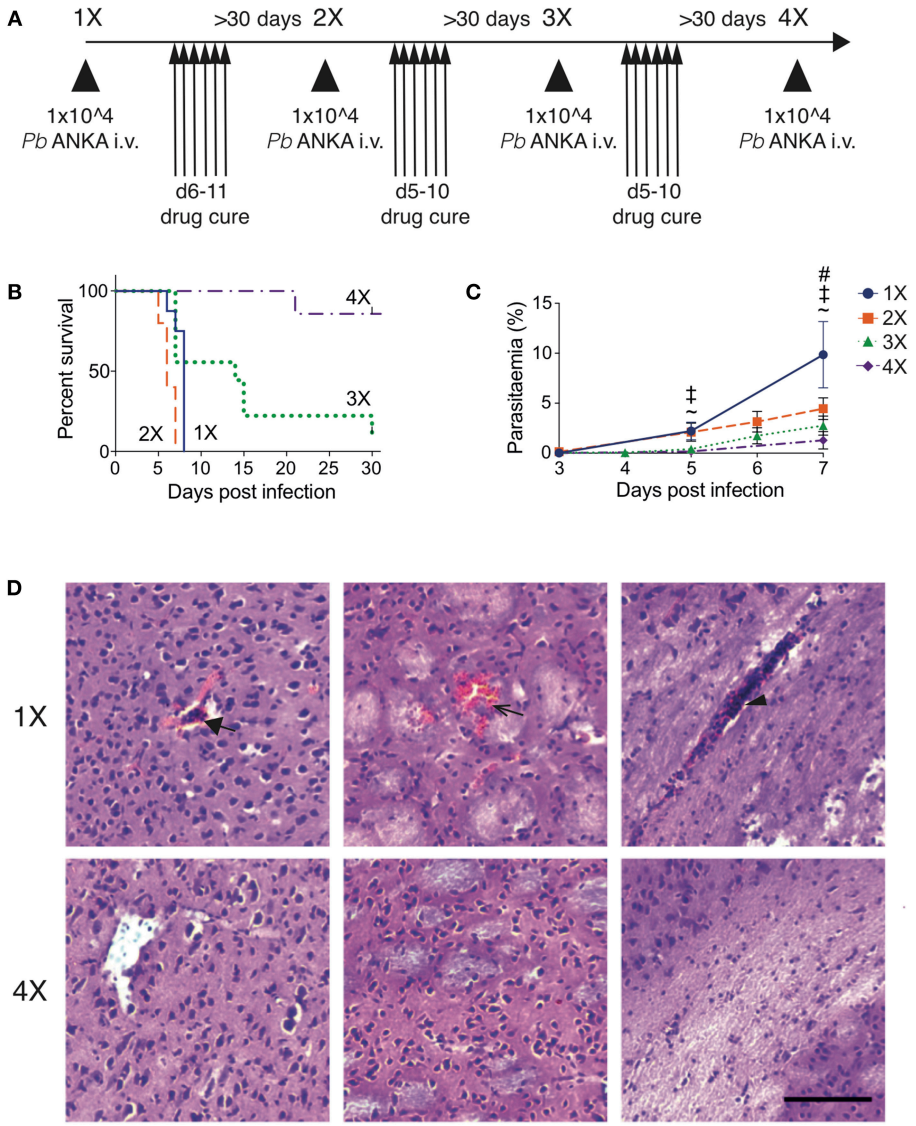

FIGURE 1 | Three rounds of infection-drug cure promote resistance to ECM in susceptible C57BL/6 mice. **(A)** Schematic of the experimental design. C57BL/6 mice were infected with PbA (10^4 pRBCs i.v.) or left uninfected. Mice were treated (i.p.) with chloroquine and artesunate on stated days post each infection, and re-infections were performed after a minimum interval of 30 days following cessation of drug treatment. **(B)** Kinetics of ECM development shown as percentage of survival of mice. $N = 7$–10 per group, pooled from two independent experiments. **(C)** Peripheral parasitaemia (% of pRBCs) ± SD in different infection groups. $N =$ 5–9 per group. Results are pooled from two experiments for the 1X, 2X, and 3X infection and from 3 experiments for the 4X infection. Kruskal-Wallis test with Dunn's multiple comparisons test was used for statistical analysis $p < 0.05$ denoted by #, for 1X v 3X, ‡ for 1X v 4X, and ~ for 2X v 4X. **(D)** Representative H & E stained cortical brain sections demonstrating presence (in 1X infected mice) and absence (in 4X infected mice) of cerebrovascular pRBCs (filled arrow), hemorrhage (unfilled arrow) and leukocyte packed vessels (arrow head) in 1 and 4X infected mice on day 8 post infection. Scale bar 100 μm.

Exposure-Induced Resistance to ECM Corresponds With Specific Inhibition of Splenic CD8$^+$ T Cell Cytolytic Capacity and Not Generalized Suppression of Splenic Effector T Cell Responses

The absence of maintained transcriptional alterations in the brain between infections suggested that infection-induced protection might be controlled at the site of T cell priming, namely the spleen. We therefore examined splenic T cells responses in 1X and 4X infected mice. The numbers of splenic CD8$^+$ and CD4$^+$ T cells were significantly increased in 4X infected mice compared with 1X infected mice on day 8 of infection, contributing to splenomegaly in 4X infected mice (**Figures 3A–D**). T cell numbers were not significantly raised in pre-4X infected mice compared with uninfected or 1X infected mice, showing that expansion of the lymphocyte populations occurred rapidly during the active fourth infection (**Figures 3A–C**).

Despite their significant expansion, CD8$^+$ T cell activation and effector function was significantly attenuated in 4X

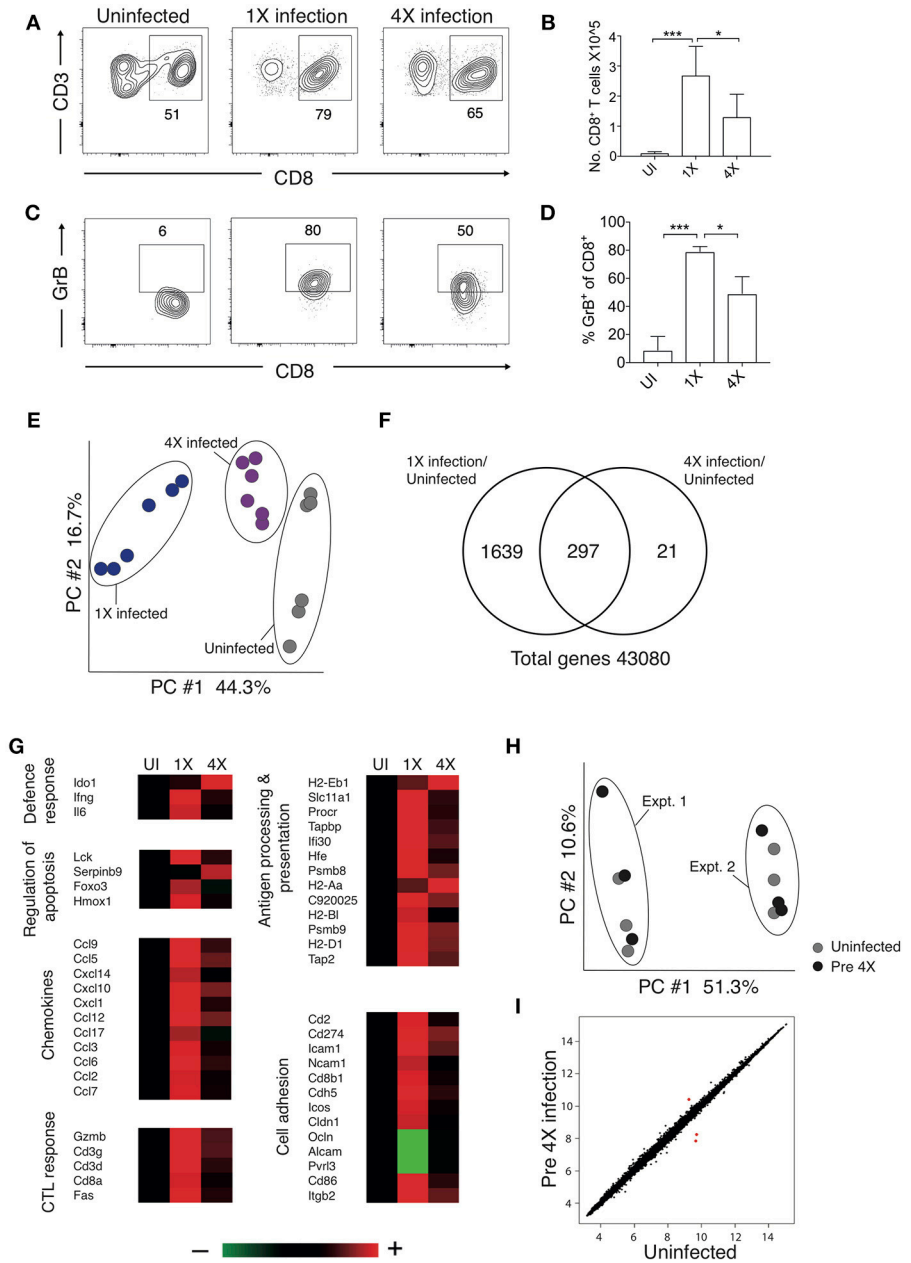

FIGURE 2 | 4X PbA infected mice have a distinct whole brain transcriptional signature **(A–D)** Perfused whole brains were removed from 4X infected and age-matched 1X infected C57BL/6 mice on day 8 p.i. (when 1X developed ECM), and age-matched naïve mice. **(A)** Representative flow cytometric plots showing identification of and **(B)** numbers of $CD8^+$ T cells in the brain (mean ± S.D.). **(C)** Representative flow cytometric plots showing granzyme B expression by and **(D)** frequencies of granzyme B expressing $CD8^+$ T cells in the brain (mean ± S.D.). **(A,C)** Numbers denote the percentage of cells within the gate. **(B,D)** $N = 4–8$ per group, pooled from two independent experiments. Statistical analyses were performed with Kruskal-Wallis test with Dunn's multiple comparisons test or with one-way ANOVA with Tukey's test, depending on normality of data ($^*p \leq 0.05$ and $^{***}p \leq 0.001$). **(E–G)** Microarray analysis was performed on perfused whole brains from 4X infected and age-matched 1X infected C57BL/6 mice on day 8 p.i. (when 1X developed ECM), and age-matched naïve mice. **(E)** Principal components analysis of whole-brain transcriptomes. $N = 6$ per group. Results are generated from the pooled array data from brains taken from two independent experiments. **(F)** Venn diagrams defining unique and overlapping genes differentially expressed between 4X infected vs. uninfected mice and 1X infected mice vs. uninfected mice. **(G)** Genes in **(F)** and **Supplementary Figure 2** were filtered to identify genes differentially expressed in brains of 1 and 4X infected mice on day 8 of infection. Heat maps showing differentially expressed genes grouped by enriched biological processes identified within DAVID bioinformatics database. Results ($n = 6$ per group) are generated from the pooled array data from brains taken from two independent experiments. **(H,I)** Perfused whole brains were removed from pre-4X infected mice (minimum 30 days post clearance of 3X infection) and age-matched uninfected mice for microarray analysis. **(H)** Principal components analysis of whole-brain transcriptomes ($n = 6$ per group). Results are generated from the pooled array data from brains taken from two independent experiments. **(I)** Scatter plot comparing gene expression between pre-4X infected mice and uninfected mice. Each data point represents the mean expression level of a gene. Results ($n = 6$ per group) are generated from the pooled array data from brains taken from two independent experiments.

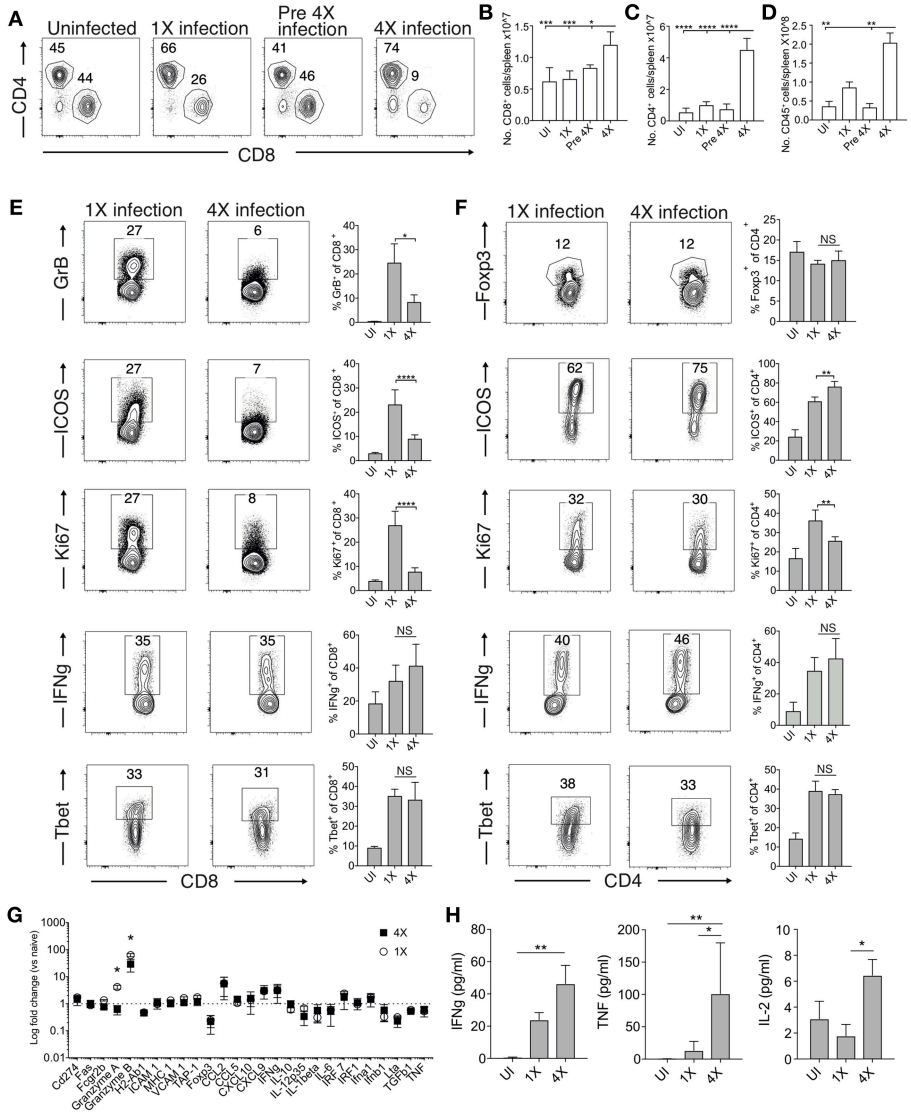

FIGURE 3 | 4X infected mice develop significantly altered splenic $CD8^+$ and $CD4^+$ T cell responses. **(A–H)** Spleens and plasma were removed from 4X infected and age-matched 1X infected C57BL/6 mice on day 8 post-infection (when 1X infected mice developed ECM), from pre-4X infected C57BL/6 mice (minimum 30 days post clearance of 3X infection), and age-matched uninfected mice. **(A)** Representative flow cytometric plots and the frequencies of splenic $CD8^+$ T cells and $CD4^+$ T cells, gated on live $CD45^+$ $CD3^+$ cells. Total numbers of splenic **(B)** $CD8^+$ T cells, **(C)** $CD4^+$ T cells and **(D)** CD45+ cells. **(E,F)** Representative flow cytometric plots and the frequencies of splenic **(E)** $CD8^+$ T cells and **(F)** $CD4^+$ T cells expressing markers of activation, function and differentiation. **(G)** Nanostring analysis of gene expression in whole spleen tissue from 4X and 1X infected mice (on day 8 of infection), expressed relative to gene expression in age-matched uninfected mice (fold change of 1 defines level of gene expression in uninfected mice). **(H)** Cytokine bead array of plasma cytokine levels in 4X, 1X infected mice and aged matched uninfected C57BL/6 mice. **(A,E,F)** Numbers denote the percentage of cells within the gate. **(B–G)** Results are the mean ± SD of the group, representative of two independent experiments with **(B–D)** $n = 3$–6 per group, **(E,F)** $n = 3$–10 per group and **(G)** $n = 3$ per group. **(H)** $N = 4$–7 per group, pooled from two independent experiments. **(B–F,H)** Statistical analyses were performed with Kruskal-Wallis test with Dunn's multiple comparisons test or with one-way ANOVA with Tukey's test, depending on normality of data ($^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$). **(G)** Statistical analysis by Student's *t*-test, with Welch's correction ($^*p \leq 0.05$).

infected mice compared with 1X infected mice, as shown by reduced expression of granzyme B, ICOS and Ki67 (**Figure 3E**). Interestingly, however, the frequencies of splenic $CD8^+$ T cells expressing T-bet and IFN-γ were similar in 4X and 1X infected mice (**Figure 3E**). In contrast, the activation and differentiation of splenic $CD4^+$ T cells was generally unaltered in 4X infected mice compared with 1X infected mice, with only a minor reduction in Ki67 and upregulation in ICOS expression by $CD4^+$ T cells in 4X infected mice (**Figure 3F**). The frequencies of $CD4^+$ T cells expressing Foxp3 was not significantly different in 4X and 1X infected mice, suggesting that altered regulatory T cell development was not the major reason for suppression of $CD8^+$ T cell activity and prevention of ECM in 4X infected mice (**Figure 3F**).

Studying the splenic and systemic immune signature in 4X infected mice compared with 1X infected mice in more detail revealed that the gene expression of granzyme B and granzyme A was significantly reduced in 4X infected mice compared with 1X infected mice (examining gene expression in whole spleen tissue), whereas there was no significant differences in expression of 25 other immune response genes, including antigen presenting molecules (TAP-1, MHC-1), pro-inflammatory chemokines (CXCL9, CXCL10, CCL2, CCL5), and cytokines (TNF, IFN-γ, LT-α) (**Figure 3G**). Moreover, plasma levels of TNF, IFN-γ and IL-2 were comparable or higher in 4X infected mice than in 1X infected mice (**Figure 3H**).

Collectively, our results, therefore, show that infection-induced resistance to ECM was associated with the specific reduction in splenic $CD8^+$ T cell cytotoxic functions and granzyme expression, rather than non-selective suppression of adaptive T cell expansion and activation, or general dampening of inflammation.

Infection-Induced Resistance Is Associated With Generation of Heterogeneous Atypical and Regulatory B Cell Populations and Improved Anti-parasite Ab Responses

Chronic and repetitive *P. falciparum* infection is associated with the development of atypical B cell populations, the functions of which are still debated (13–19). As well as being potentially important sources of anti-parasite Ab (15), they may also exert immune regulatory activity, suppressing inflammation (14, 17–19). Consequently, we investigated whether repeated PbA infection led to the formation of atypical B cell populations and how this may contribute to infection-induced resistance to ECM. The numbers of splenic $CD19^+$ B cells were significantly increased in the 4X infected mice compared with 1X infected mice, examined on day 8 of infection (**Figures 4A,B**). Whilst there was a large increase in the frequencies of $CD19^+CD138^+$ (putatively plasmablasts) and $CD19^+CD80^+$ [activated and memory B cells (33)] in 4X infected mice compared with 1X infected mice, we also observed an increase in the frequencies of atypical $CD19^+$ cells expressing CD11c [B cells that exert APC activity and may contribute to autoimmunity (13, 34–36)], PDCA-1 [suppressor B cells but which also produce Abs (37)] and PDL-1 [putatively regulatory B cells (38)] in 4X infected mice compared with 1X infected mice (**Figure 4C**).

The frequencies and numbers of class-switched IgD^-IgM^- B cells were also significantly increased in 4X infected mice compared with 1X infected mice (**Figure 4D**), which corresponded with significantly higher plasma titers of anti-*Pb*$MSP1_{19}$ and total anti-PbA IgG antibodies in 4X infected mice than 1X infected mice (**Figures 4E,F**). Of interest, total anti-PbA Ab responses developed much more gradually upon repeated infections than anti- *Pb*$MSP1_{19}$ Ab responses (which largely peaked during 2X infection when mice were still highly susceptible to ECM), (**Figures 4E–G**). The levels of anti-*Pb*$MSP1_{19}$ IgG were not boosted at all during the fourth infection (when measured on day 8 of infection and compared with levels in pre-4X infected mice), whereas the levels of total PbA-specific IgG were boosted only slightly during the 4th infection (**Figures 4E–G**).

Thus, infection-induced resistance was associated with the generation of a heterogeneous B cell response and elevated anti-parasite IgG production, suggesting potential roles for atypical and/or regulatory B cells in promoting resistance to ECM.

Individual and Transitory Manipulation of Antibody and Atypical B Cell Populations Does Not Alter Resistance or Susceptibility to ECM

To examine the importance of atypical B cell populations in infection-induced resistance to ECM, we treated mice immediately prior to and during 4X infection with anti-CD20mAb (to enable pan-B cell depletion). This approach was necessary due to the spectrum of cell populations that express atypical B cell markers, precluding the use of other specific antibody-targeting approaches. Anti-CD20 mAb depleted nearly all $CD19^+$ B cells in 4X infected mice (**Figure 5A**); including the majority of class switched $CD19^+$ B cells (**Figure 5B**). However, as previously reported (39), treatment had less of an effect on $CD19^-CD138^+$ plasma cells than on putative regulatory and antigen-presenting B cells (including $CD19^+$PD-$L1^+$, $CD19^+CD11c^+$, $CD19^+$PDCA-1^+, and $CD19^+$MHC-II^+ B cells), $CD19^+CD138^+$ plasmablasts and $CD19^+CD80^+$ activated / memory cells (**Figure 5C**). Nevertheless, despite the depletion of the majority of the B cell compartment, anti-CD20 mAb treatment did not increase the activation or function of splenic or intracerebral $CD8^+$ T cells during 4th infection (**Figures 5D,E**), with granzyme B expression by $CD8^+$ T cells in anti-CD20mAb and control Ab treated 4X infected mice being significantly lower than in 1X infection (**Figure S4**). Consequently, anti-CD20 mAb administration did not alter the resistance of 4X infected mice to ECM (**Figures 5F,G**). In agreement with this, blockade of IL-10 activity, a major mechanism of Breg suppression (38), did not reverse the resistance of 4X infected mice to ECM (**Figure S4**). This is despite the fact that IL-10 plasma levels trended higher in 4X infected mice compared with 1X infected mice (**Figure S4**), and adoptive transfer of IL-10 producing Bregs from repeatedly infected mice has previously been shown to promote resistance to ECM in primary infected mice (40).

The apparent redundancy of infection-induced atypical B cells (and other B cell populations) in actively mediating infection-induced resistance to ECM during the 4X infection suggested that circulating anti-parasite antibody (which was maintained at high level in pre-4X infected mice; **Figures 4E,F**), may be sufficient to confer protection against ECM. To investigate whether this was the case, we performed a passive transfer experiment by transferring plasma from 4X infected mice containing high titres of anti-parasite IgG (defined in **Figures 4E,F**) into mice during the course of 1X infection. Despite significantly lowering peripheral parasite levels during the early phases of 1X infection, passive transfer of plasma obtained from repeatedly infected mice (through 4 separate injections during the course of infection) failed to protect 1X infected mice from ECM (**Figures 5H,I**), or reduce $CD8^+$ T cell activation within the spleen or brain (**Figures 5J,K**). Consequently, high levels of

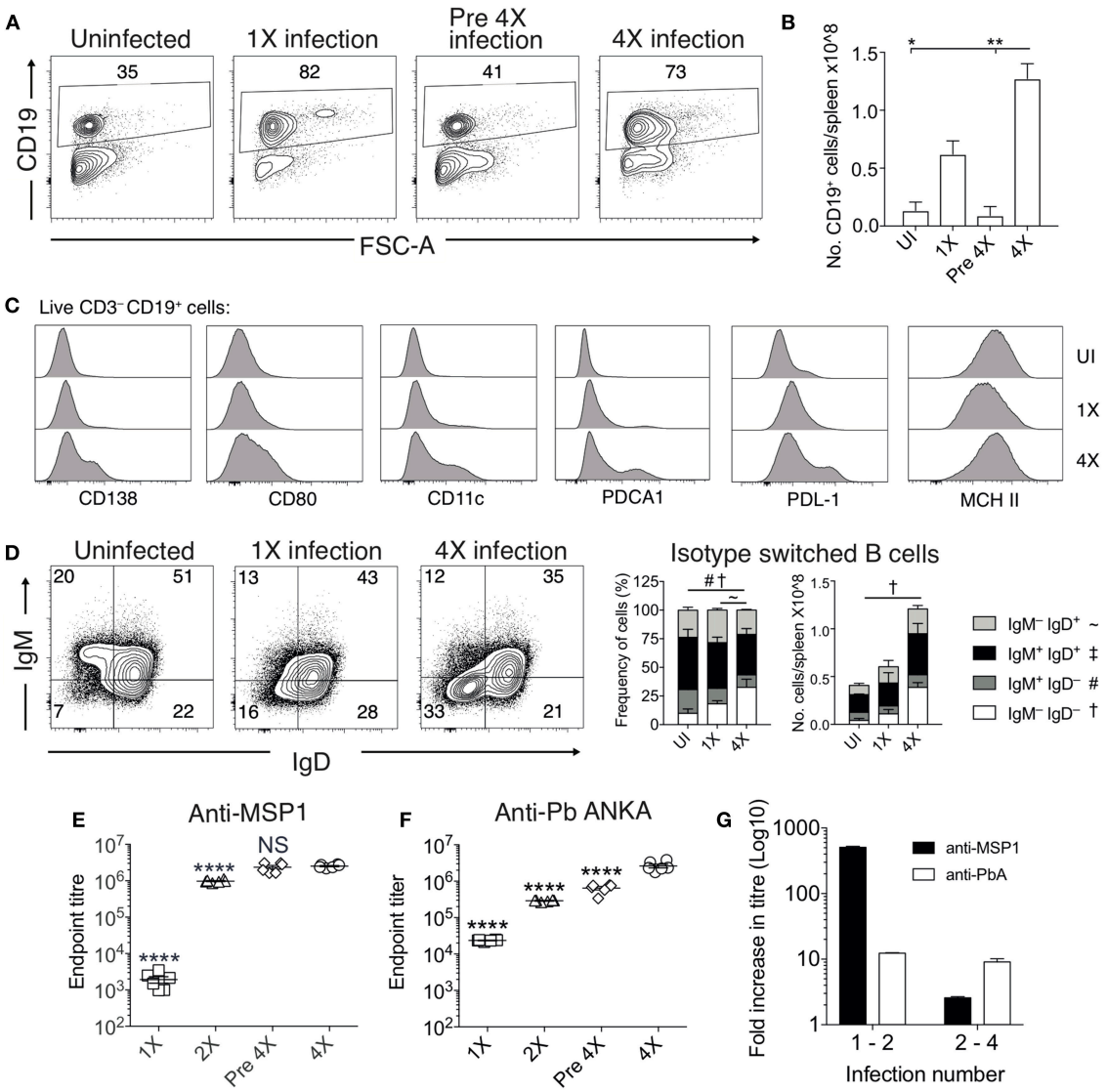

FIGURE 4 | Anti-parasite IgG titres and atypical B cell populations are significantly increased in 4X infected mice. **(A–G)** Spleens and plasma were obtained from 4X infected and age-matched 1X infected C57BL/6 mice on day 8 post-infection (when 1X infected mice developed ECM), from pre 4X infected mice (minimum 30 days post-clearance of 3X infection) and from age-matched uninfected mice. **(A)** Representative flow cytometric plots and the frequencies of splenic $CD19^+$ B cells, gated on live $CD45^+$ $CD3^-$ cells. Numbers denote the percentage of cells within the gate. **(B)** Total numbers of splenic $CD19^+$ B cells. **(C)** Expression of CD138, CD80, CD11c, PDCA1, PDL-1, and MHC II by $CD19^+$ splenic B cells from uninfected, 1X infected and 4X infected mice. **(D)** Left, Representative flow cytometric plots (gated on live $CD45^+CD3^-CD19^+$ cells), right, the frequencies and numbers of non-switched (IgM^+IgD^+ and IgM^-IgD^+) and switched (IgM^+IgD^-, and IgM^-IgD^-) splenic $CD19^+$ B cells. The end point plasma titres of **(E)** anti- $PbMSP1_{19}$ and **(F)** anti-PbA IgG, with background titer from naïve mice subtracted. **(G)** Graphical representation of the fold change in Ab titer between 1X and 2X infected mice and between 2X and 4X infected mice. **(B,D)** $N = 2$–6 per group, representative of two independent experiments (mean ± SD). Statistical analyses were performed with Kruskal-Wallis test with Dunn's multiple comparisons test $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $p < 0.05$ denoted by #, ~, † for indicated groups. **(E,F)** $N = 6$ per group, pooled from two independent experiments (mean ± SD). Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparisons test, shown for 4X infected mice compared to each other group ($^{****}p \leq 0.0001$).

anti-parasite antibody did not appear to be sufficient to confer protection against ECM.

Mice Unable to Produce Secreted Antibody Fail to Acquire Infection-Induced Resistance to ECM

The above results suggested that anti-parasite Ab may not be sufficient for active protection against ECM when given acutely during a primary infection. Antibody can directly and indirectly coordinate many events within the immune system, potentially influencing long-term immunity and controlling the nature of immune responses during challenge infections (41–44). As such, we reasoned that antibody may need to be produced and maintained during the initial rounds of PbA infection to condition (or educate) the immune system in repeatedly infected

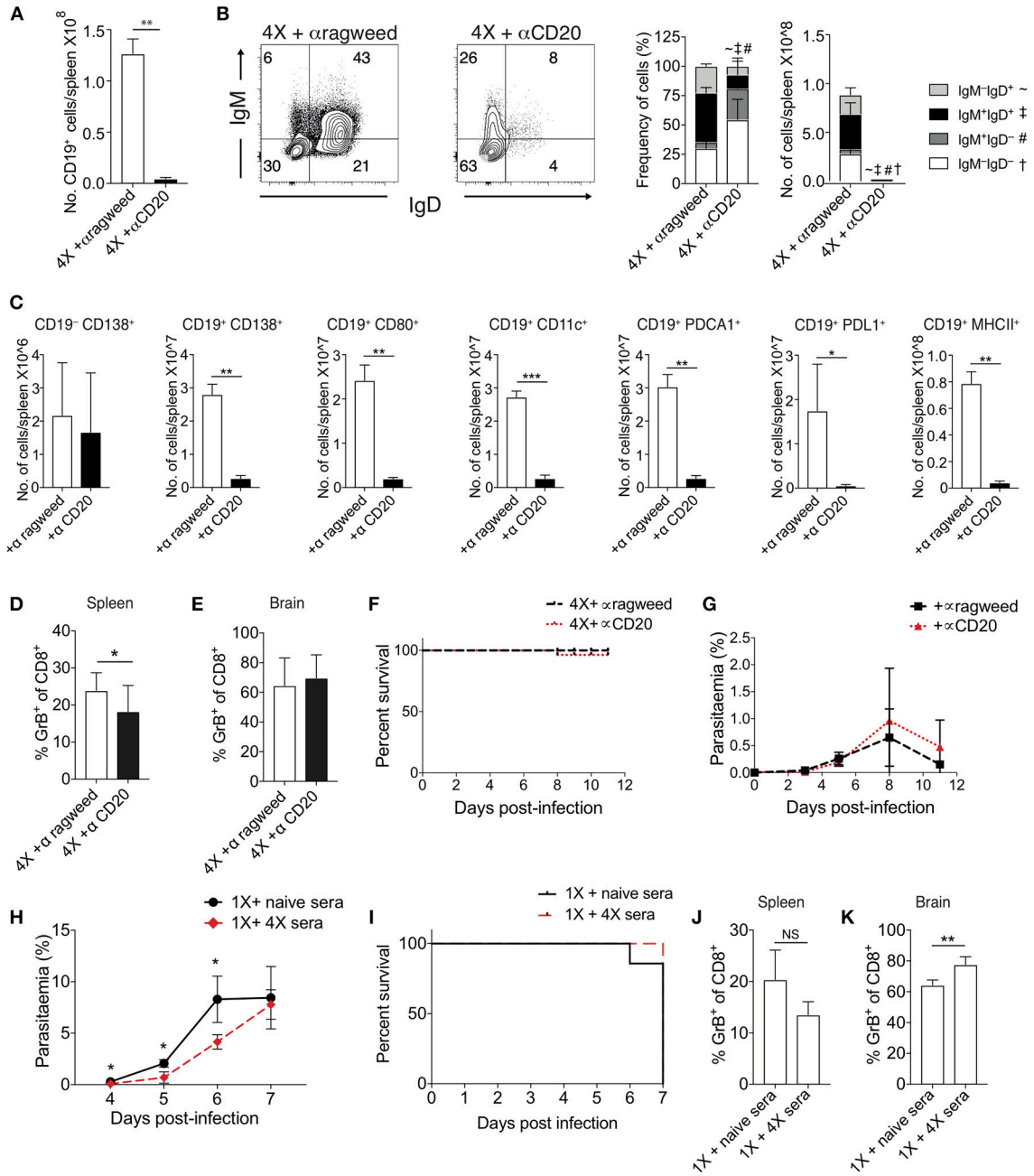

FIGURE 5 | Ablation of B cell populations or transitory boosting of anti-parasite antibody does not alter dynamics of ECM in 1X or 4X infected mice. **(A–G)** C57BL/6 mice were injected (i.p) one day prior to 4X infection and on days 2, 5, 8, 11 of infection, with either (250 μg) anti-CD20 mAb or (250 μg) control anti-ragweed mAb. **(A)** Total numbers of splenic $CD19^+$ B cells. **(B)** Left, Representative flow cytometric plots (gated on live $CD45^+CD3^-CD19^+$ cells), right, the frequencies of non-switched (IgM^+IgD^+ and IgM^-IgD^+) and switched (IgM^+IgD^-, and IgM^-IgD^-) splenic $CD19^+$ B cells. **(C)** Total numbers of splenic $CD19^-$ $CD138^+$ plasma B cells and splenic $CD19^+$ B cells expressing CD138, CD80, CD11c, PDCA1, PDL-1, and MHC II in 4X infected mice that received anti-CD20 mAb or anti-ragweed mAb. **(D,E)** The frequencies of Granzyme B expressing $CD8^+$ T cells in the **(D)** spleen and **(E)** brain in 4X infected mice that received anti-CD20 mAb or anti-ragweed mAb. **(F)** Survival of 4X infected mice given anti-CD20mAb or control mAb. **(G)** Parasitaemia (% of pRBCs) of 4X infected mice given anti-CD20mAb or control mAb. **(H–K)** C57BL/6 mice received 500 μl of heat-inactivated plasma (obtained from 4X infected mice or, as a control, from age-matched naïve mice) prior to and during 1X infection (4 i.p. injections between day −1 and day 5 of infection). **(H)** Peripheral parasitaemia (% of pRBCs) in 4X infected mice that received plasma from 4X or uninfected mice **(I)** Kinetics of ECM development shown as percentage survival of 1X infected mice that received plasma from 4X or uninfected mice. **(J,K)** The frequencies of granzyme B expressing $CD8^+$ T cells in the **(J)** spleen and **(K)** brain in 1X infected mice that received plasma from 4X or naïve mice. **(A–K)** Results are the mean ± SD of the group. **(A–C)** $N = 3$–4 per group, representative of two independent experiments. **(D,E)** $N = 7$–8 per group, pooled from two independent experiments. **(F, G)** $N = 10$–13 per group, pooled from two independent experiments. **(H, I)** $N = 6$–7 per group**,** pooled from two independent experiments. **(J,K)** $N = 4$, representative of two independent experiments. Statistical analyses were performed with by Student's *t*-test, with Welch's correction or Mann-Whitney test depending on normality of data ($^*p \leq 0.05$, $^{**}p \leq 0.01$ $^{***}p \leq 0.001$ and $p < 0.05$ denoted by #, ∼, ‡ for indicated groups).

mice, eventually promoting resistance to ECM in 4X infected mice. To investigate if this hypothesis was correct, we used IgMi mice, which contain B cells that are able to produce membrane IgM but are unable to make secreted antibody or undergo class switching (21, 22), As expected, no anti- *Pb*MSP1$_{19}$ or total anti-PbA IgG was detected in the plasma of 4X infected IgMi mice. Importantly, and in contrast to littermate WT mice, 4X infected IgMi mice failed to acquire complete or even partial resistance to ECM, with 100% (8/8) of mice developing accelerated late-stage ECM between days 6 and 7 p.i. (**Figure 6A**). Parasite control was reduced but not completely abrogated in 4X infected IgMi mice compared with 1X infected IgMi mice and 4X infected WT mice (**Figure 6B**).

As expected, essentially all CD19$^+$ B cells in IgMi mice expressed IgM, but not IgD, during 1X infection and this expression pattern did not change during 4X infection (**Figure 6C**). Interestingly, although redundant for active resistance during 4X infection in WT mice, the development of atypical and/or regulatory CD11c$^+$, PDCA-1$^+$ and PDL-1$^+$ B cell populations, as well as CD138$^+$ and CD80$^+$ populations, were abrogated in 4X IgMi mice (**Figure 6D**), indicating that infection-induced development of atypical B cell populations during malaria was dependent upon secreted antibody.

Splenic CD8$^+$ T cell responses were significantly increased in 4X IgMi mice compared with 4X infected WT mice, as demonstrated by the significantly increased frequencies of CD8$^+$ T cells expressing granzyme B, ICOS, Ki67, and KLRG1 in 4X infected IgMi mice compared with 4X infected WT mice (**Figure 6E**). Moreover, the frequencies of CD8$^+$ T cells expressing granzyme B were higher in the brains of 4X infected IgMi mice compared with 4X WT mice (**Figure 6F**). Interestingly, despite lower peripheral parasitaemia, the expression of granzyme B and KLRG1 by splenic CD8$^+$ T cells was enhanced rather than decreased in 4X compared with 1X infected IgMi mice, suggesting that that CD8$^+$ T cell activation during repeated PbA infections was not directly proportional to peripheral parasite burden (**Figures 6B,E**). In contrast, the activation of splenic CD4$^+$ T cell responses were unaltered in 4X IgMi mice compared with corresponding 4X infected WT mice (**Figure S5**).

Thus, collectively, our data suggest that secreted antibody is required during the repeated rounds of infection to modulate the B cell compartment and suppress pathogenic CD8$^+$ T cell responses to establish infection-induced resistance to ECM.

DISCUSSION

In this study, we have shown that multiple rounds of PbA parasite exposure lead to the incremental development of ECM resistance in otherwise susceptible C57BL/6 background mice. Our results, therefore, appear to recapitulate the development of naturally acquired resistance to CM in humans in malarial endemic regions (4). Although protection against ECM was associated with the gradual control of peripheral parasite burdens and reduced parasite accumulation within the brain, sterile immunity was not achieved.

In this model system infection-induced protection against ECM appeared to depend upon the inhibition of memory CD8$^+$ T cell reactivation, rather than a generalized dampening of inflammation. Indeed, splenic CD4$^+$ T cell responses and plasma pro-inflammatory cytokine levels were comparable or increased in 4X infected mice compared with 1X infected mice. Granzyme B expression by CD8$^+$ T cells was reduced in 4X infected mice compared with 1X infected mice but IFN-γ production was not significantly affected by the number of infections. It has been shown that IL-12/IL-18 and IL-15 differentially control IFN-γ production and granzyme B expression by reactivating memory CD8$^+$ T cells, respectively (45). Consequently, it is foreseeable that modified memory CD8$^+$ T cell reactivation during 4X infection was caused by alterations in the levels of IL-15. However, it is possible that lowered peripheral parasitaemia in 4X infected mice also contributed to alterations in CD8$^+$ T cell reactivation.

It is probable that the inhibition of CD8$^+$ T cell pathogenic responses in the brain of 4X infected mice was caused by the modulation of CD8$^+$ T cell immune activation within the spleen, leading to reduced migration of less-pathogenic CD8$^+$ T cells to the brain in 4X infected mice. Nevertheless, our transcriptomics analyses also suggest that many of the tissue signals, such as endothelial cell cross presentation of parasite antigen, which are necessary for CD8$^+$ T cells to arrest, mediate damage to the blood brain barrier and provoke ECM (32, 46, 47), were also down regulated in the brains of 4X infected mice. This was likely, in part, due to the fact that parasite accumulation appeared, through qualitative assessment, to be reduced / prevented in brains of 4X compared with 1X infected mice. Indeed, it has previously been shown that pathogenic CD8$^+$ T cell activation in the brain during *PbA* infection depends upon a threshold level of parasite accumulation and parasitic antigen in the brain (48). Interestingly, our transcriptomics analyses suggests that infection-induced dampening of intracerebral immune responses and resistance to ECM mirrors processes that specify genetic resistance to ECM, and those associated with avirulent parasite infections (49–51). The datasets generated in this study will be useful resources to identify other gene candidates that control resistance to ECM during *Pb*A infection.

In terms of the infection-induced processes that provided protection against ECM, we have shown the critical non-redundant role of antibody. IgMi mice, which were unable to produce secreted antibody or undergo class switching (21, 22), developed hyperactive splenic and intracerebral CD8$^+$ T cell responses during 4X infection and consequently failed to acquire infection-induced resistance to ECM. Antibody responses to the immunodominant antigen MSP-1$_{19}$ did not correlate with resistance to ECM; however, total anti-parasite Ab responses developed more gradually over repeated infections and were eventually maintained, at least short term, between 3rd and 4th infections. These observations are in agreement with previous studies showing that anti- *Pb*MSP1 antibody titres did not correspond with vaccine induced protection against PbA (30). Also, they are consistent with studies in human malaria, showing that exposure-induced protection against *P. falciparum* is associated with the gradual accumulation of B cells

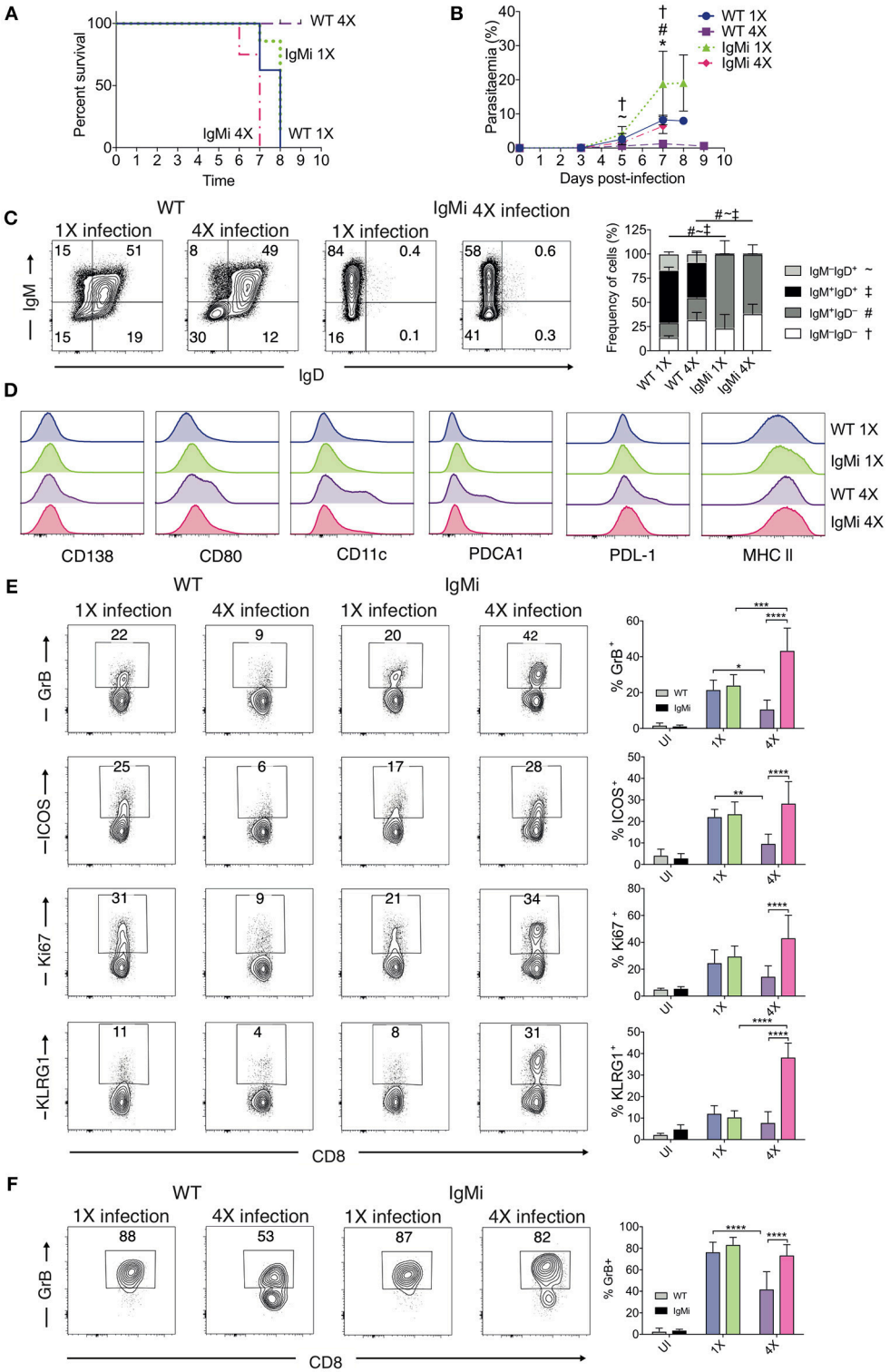

FIGURE 6 | IgMi mice do not develop infection-induced resistance to ECM. **(A–F)** IgMi mice and WT littermate controls were infected with PbA (10^4 pRBCs i.v.) or left uninfected. Mice were treated (i.p.) with chloroquine and artesunate from day 5 or 6 post each infection, and re-infections were performed after a minimum interval of 30 days following cessation of drug treatment. **(A)** Kinetics of ECM development, shown as percentage of survival of mice. **(B)** Peripheral parasitaemia (% of pRBCs). **(C)** Left, Representative flow cytometric plots (gated on live $CD45^+CD3^-CD19^+$ cells), right, the frequencies of non-switched (IgM^+IgD^+ and IgM^-IgD^+) and switched (IgM^+IgD^-, and IgM^-IgD^-) splenic $CD19^+$ B cells. **(D)** Expression of CD138, CD80, CD11c, PDCA1, PDL-1 and MHC II by $CD19^+$ splenic B cells from 1X

(Continued)

FIGURE 6 | infected and 4X infected WT and IgMi mice. **(E,F)** Activation phenotype of **(E)** splenic and **(F)** brain accumulating $CD8^+$ T cells. **(B,C,E,F)** Results are the mean ± SD of the group. **(A,B)** $N = 7–8$ per group, pooled from two independent experiments. **(C)** $N = 3–4$ per group, representative of two independent experiments. **(E,F)** $N = 5–8$ per group, pooled from two independent experiments. **(B,E,F)** Statistical analyses were performed with Kruskal-Wallis test with Dunn's multiple comparisons test or with one-way ANOVA with Tukey's test depending on normality of data (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, and $p < 0.05$ denoted as follows *, WT 1X v IgMi 1X, ∼, WT 4X v IgMi 1X, #, WT 4X v IgMi 4X, †, IgMi 1X v IgMi 4X). **(C)** Statistical analysis by Student's t-test, with Welch's correction for indicated groups ($p < 0.05$ denoted by #, ∼, ‡ for the indicated groups).

with different specificities (12, 52, 53). The role of maintained antibody in infection-induced protection to ECM is supported by seroepidemiological studies in humans, where the level of anti-*P. falciparum* antibody prior to the malaria season, but not after the malaria season, correlated with protection against clinical disease (12, 52).

The fact that IgMi mice failed to develop resistance to ECM but we were unable to protect 1X infected mice by passive transfer of plasma from 4X infected mice may suggest that we failed to transfer enough antibody in our experiments to promote protection. Whilst we cannot fully discount this possibility, passive transfer of plasma from 4X infected mice did lead to significant reductions in parasite levels during the course of 1X infection. Moreover, we transferred higher amounts of antibody (four injections of 500 μl of plasma with Ab end point titer $>1 \times 10^6$ during the course of infection) than has been used to significantly reduce parasite burdens and protect mice from other malarial species, such as *P. yoelii* and *P. chabaudi* (31, 54). The inability of passively transferred anti-parasite Ab to provide protection against *P. berghei* infection has previously been described (54) and, to date, we are unaware of any other study where passive transfer of antisera has promoted resistance against ECM in primary PbA infected mice. Thus, our data argue that repeated PbA infection promotes generation and maintenance of anti-parasite antibodies that establish changes in the immune system over the course of repeat infections that provide protection against ECM, and which cannot be easily recapitulated by acute administration of high titres of antibody to primary infected mice.

There are various potential ways through which antibody could educate the immune system during the course of repetitive *Plasmodium* infections to inhibit $CD8^+$ T cell activity and promote resistance to ECM. Antibody, in the form of immune complexes and via triggering Fc receptors, or through activating complement, can significantly modulate APC activation, cytokine production and antigen presentation (41–44). There is accumulating evidence that memory $CD8^+$ T cell reactivation can be substantially influenced by the repertoire, duration and level of cytokine production, co-stimulation, and nature of antigen presentation from different APC populations (45, 55–57). For example, in viral infections antibody-antigen immune complexes can extend the duration of antigen presentation post-clearance of infection, which although not affecting the ultimate magnitude of the maintained memory $CD8^+$ T cell population, significantly influences memory $CD8^+$ T cell reactivation (41). Notably, modulation of innate cell phenotype and function can persist post-clearance of *Plasmodium* infection (58), and the duration of these changes have been linked to the maintenance of anti-parasite antibody (59). It has also been reported that the $CD11c^+$ DC population is altered in mice repeatedly infected with PbA and that this contributes to resistance to ECM (60). Thus, antibody may extend infection-induced alterations in innate cell activity and antigen presentation following clearance of infection, as well as influencing APC reactivity in the acute period during challenge infections, ultimately affecting memory $CD8^+$ T cell reactivation. Although we focussed on anti-parasite IgG in this study, it has been shown anti-malarial memory B cell populations maintain the capacity for IgM production (61). IgM through binding to TOSO on B cells has been shown to indirectly regulate $CD8^+$ T cell activation during PbA infection (62). Thus, secreted IgM and IgG isotypes may play a role in regulating memory $CD8^+$ T cell reactivation during repeated *Plasmodium* spp. infection. Defining precisely how antibody, and importantly gradual acquisition of different specificities of anti-parasite Ab, influence the immune environment to repress pathogenic $CD8^+$ T cell responses during 4X PbA infection to promote resistance to ECM should be the subject of detailed investigations in further studies. For example, the role of the Fcgamma RIIb receptor, which is expressed on $CD8^+$ T cells and which influences memory $CD8^+$ T cell activation during Listeria monocytogenes infection (63), could be examined by studying whether Fcgamma RIIb deficient mice develop infection-inducedresistance to ECM.

Whilst we believe antibody was key for protection in our infection-drug cure model, it was of interest that infection-induced protection against ECM also corresponded with the formation of atypical B cell populations in the spleen, and that development of these atypical B cell populations was abrogated in repeatedly infected IgMi mice. The function of atypical B cell populations during malaria, which have principally be defined as memory B cells (12–15), but in our model could also include non-conforming plasmablasts and plasma cells (16, 33, 34), is still unclear. Very recently it has been shown that atypical B cells are short-lived activated B cells during malaria that form as part of a natural ongoing B cell response (64). Evidence suggests atypical B cells may be protective through anti-parasite Ab production (15) or via immunoregulatory activity, suppressing damaging inflammation (14, 17–19), or they are dysfunctional, impeding generation of long-lived humoral immunity or contributing to autoimmunity (12–16, 19). We found that transitory depletion of the majority of atypical B cells as well as $CD19^+IgM^+IgD^+$ cells [which include classical regulatory B cells (38)] immediately prior to and during 4X infection by anti-CD20 mAb administration failed to reverse infection-induced resistance to ECM. This showed that atypical B cells and regulatory B cell populations (as well as other classical B cell populations) were not necessary for active protection to ECM during the on-going 4X infection, at least when the duration between 3 and 4X infection was relatively short. This result contrasts with the previous observation that adoptive

transfer of IL-10 expressing Bregs from ECM resistant multiply infected mice promotes resistance to ECM in primary infected mice (40). Whilst the reason for our conflicting data is unclear it is probable that although high numbers of IL-10 expressing Bregs can be sufficient for protection against ECM following adoptive transfer, they are not necessary for physiological active protection in the context of a full working memory immune compartment. In agreement with this, we found that IL-10R blockade, although slightly improving parasite control, did not affect the resistance of 4X infected WT mice to ECM. Nevertheless, we cannot discount the possibility that the atypical B cell populations, in concert with antibody, may play a role in gradually modulating the immune system during repetitive infections, eventually conditioning an immunological state that provides resistance to ECM in 4X infected mice. Indeed, B cells can undertake APC functions and can directly tolerise $CD8^+$ T cells (65). The overall role of B cells in establishing anti-disease immunity could be examined in the future using uMT mice.

In summary, in this model of repeated parasite exposure we have shown that infection-induced resistance to ECM was associated with the repression of memory $CD8^+$ T cell reactivation during 4X infection. Resistance to ECM and inhibition of pathogenic memory $CD8^+$ T cell responses was ablated in mice unable to produce secreted antibody but protection against ECM could not be recapitulated solely by the transfer of high titer anti-parasite antibody prior to primary infection, and protection during the fourth infection was not reversed by active depletion of the majority of the B cell compartment. Thus, our results suggest that anti-parasite Ab may gradually orchestrate and maintain changes in the immunological environment that eventually attenuate splenic memory $CD8^+$ T cell reactivation in repeatedly infected mice. Although the contribution of $CD8^+$ T cells in the pathogenesis of HCM remains controversial, there is some evidence that activated $CD8^+$ T cells may play a role in severe malarial disease (66, 67). Consequently, our results provide insight into the mechanisms of protection to ECM and may have implications for studying the basis of infection-induced resistance to severe malarial disease and specifically cerebral malaria in humans.

Ethics

Animal work was approved following local ethical review by the Universities of Manchester and Oxford Animal Procedures and Ethics Committees and was performed in strict accordance with the U. K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O Project Licenses 70/7293, 30/2889, and P8829D3B4).

DATA AVAILABILITY

The microarray datasets reported in this paper have been deposited in the ArrayExpress database (accession number E-MTAB-5513).

AUTHOR CONTRIBUTIONS

TS and KC: conceptualization; TS, CI, PS, DP, SD, and KC: methodology; TS, CI, DP, PS, and AV-M: investigation; LZ: formal Analysis; TS and KC: writing—Original Draft; TS and KC: writing—review and editing; TS and KC: funding acquisition; SD and KE: resources; KC: supervision; TS: visualization.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00248/full#supplementary-material

Figure S1 | (A) Peripheral parasitaemia (% of pRBCs) ± SD in 3X and 4X infection groups from day 8 post infection ($n = 2$–10 per time point). **(B)** Representative H & E stained brain sections demonstrating presence and absence of hemorrhage, packed vessels and oedema in 1X and 4X infected mice on day 8 post infection. **(C)** Representative immunofluorescence stained cortical brain sections demonstrating presence and absence of pRBCs (green) in the $CD31^+$ cerebrovascular (red) of 1 and 4X infected mice on day 8 post infection. Nuclei were counterstained with dapi (blue).Scale bar 25 μm.

Figure S2 | (A–D) Perfused whole brains were removed from 4X infected and age-matched 1X infected C57BL/6 mice on day 8 p.i. (when 1X developed ECM), and age-matched naïve mice, for microarray analysis. **(A)** K-means and hierarchical clustering of differentially expressed genes in 4X mice vs. uninfected mice and 1X mice vs. uninfected mice. Each probe-set expression level was normalized to the naïve average. **(B)** Gene ontology analysis identifying enriched biological processes within each gene cluster, identified within DAVID bioinformatics database. **(C)** Full size defense response and **(D)** regulation of apoptosis gene ontology pathways differentially expressed in brains of 1X and 4X infected mice. $N = 6$ per group. Results are generated from the pooled array data from brains taken from two independent experiments.

Figure S3 | (A,B) Perfused whole brains were removed from 4X infected and age-matched 1X infected C57BL/6 mice on day 8 p.i. (when 1X developed ECM), for microarray analysis. Ingenuity analysis identified **(A)** IL-6- and **(B)** IFN-γ-controlled gene networks as two major pro-inflammatory gene networks downregulated in the brains of 4X infected mice compared with 1X infected mice (green color represents down-regulated gene expression and red color represents up-regulated gene expression). **(C)** Nanostring validation of expression of selected genes in whole brains of 1 and 4X infected mice on day 8 of infection (presented as fold change in expression compared with naïve brains). **(A,B)** $N = 6$ per group. Results are generated from the pooled array data from brains taken from two independent experiments. **(C)** $N = 5$ per group, from two pooled experiments. Statistical analysis by Student's *t*-test, with Welch's correction ($^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$).

Figure S4 | (A,B) C57BL/6 mice were injected (i.p) one day prior to 4X infection and on days 2, 5, 8, 11 of infection, with either (250 μg) anti-CD20 mAb or (250 μg) control anti-ragweed mAb. Frequencies of granzyme B expressing $CD8^+$ T cells in **(A)** the spleen and **(B)** the brain on day 8 post infection of age matched

naïve, 1X infected and 4X infected mice, that received anti-CD20 mAb or anti-ragweed mAb. **(C)** Cytokine bead array of plasma cytokine IL-10 levels in 4X, 1X infected mice and aged matched uninfected C57BL/6 mice. **(D)** C57BL/6 mice were injected (i.p) one day prior to the 4X infection and on every other day of 4X infection with anti-IL-10R mAb or PBS. Kinetics of ECM development shown as percentage survival of mice. **(A–C)** Results are the mean ± SD of the group. **(A,B)** $N = 4$–8 per group, pooled from two independent experiments. **(C)** $N = 4$–7 per group, pooled from two independent experiments. **(D)** $N = 9$ per group, pooled from two independent experiments. Statistical analyses were performed with Kruskal-Wallis test with Dunn's multiple comparisons test (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$).

Figure S5 | IgMi mice and WT littermate controls were infected with PbA (10^4 pRBCs i.v.) or left uninfected. Mice were treated (i.p.) with chloroquine and artesunate from day 5 or 6 post each infection, and re-infections were performed after a minimum interval of 30 days following cessation of drug treatment. Activation phenotype of splenic $CD4^+$ T cells in the different groups of IgMi and WT littermate mice. $N = 2$–4 per group, representative of two independent experiments. Statistical analyses were performed with Kruskal-Wallis test with Dunn's multiple comparisons test (*$p \leq 0.05$).

Supplementary Table 1 | C57BL/6 mice were infected with PbA (104 pRBCs i.v.) or left uninfected. Mice were treated (i.p.) with chloroquine and artesunate as shown in **Figure 1A**, and re-infections were performed after a minimum interval of 30 days following cessation of drug treatment. Table shows the day post infection, number of mice, mean peripheral parasitaemia (% of pRBCs) ± SD in different infection groups. Results are pooled from two experiments for the 1X, 2X, and 3X infection and from 3 experiments for the 4X infection.

Supplementary Table 2 | List of differentially expressed genes included within **Figure 2** and **Figure S2**.

Supplementary Table 3 | Genes in **Supplementary Table 1** filtered to identify genes differentially expressed between 4X and 1X brains.

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