HETEROLOGOUS IMMUNITY: IMPLICATIONS AND APPLICATIONS IN VACCINES AND IMMUNOTHERAPIES

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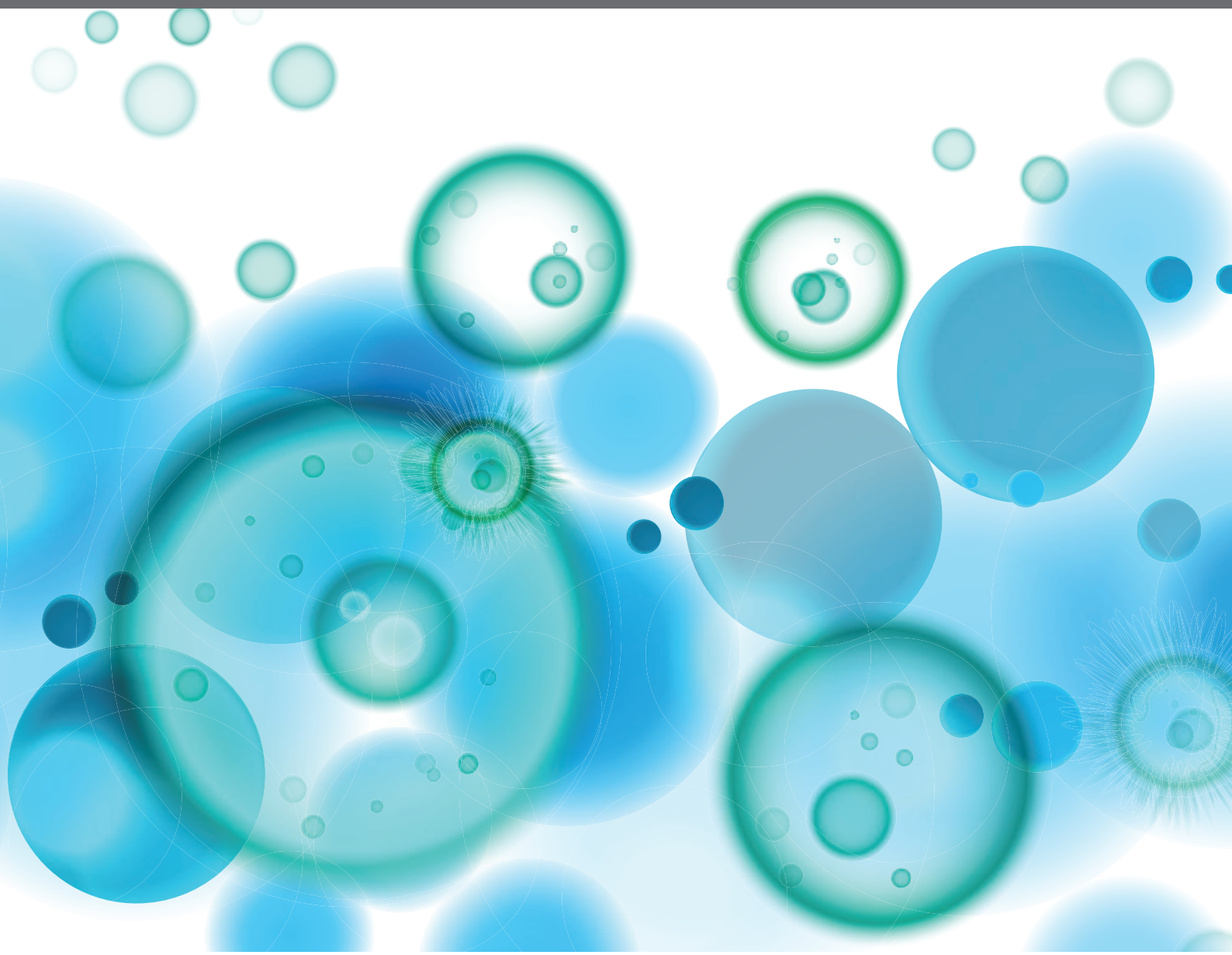

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HETEROLOGOUS IMMUNITY: IMPLICATIONS AND APPLICATIONS IN VACCINES AND IMMUNOTHERAPIES

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EDITORIAL
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Editorial: Heterologous Immunity: Implications and Applications in Vaccines and Immunotherapies

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Editorial on the Research Topic

Heterologous Immunity: Implications and Applications in Vaccines and Immunotherapies

Heterologous immunity is defined as immunity that can be induced by a pathogen or antigen against another unrelated pathogen, antigen or even an autologous antigen. The applications of "heterologous immunity" can be traced back to the first vaccine by Edward Jenner in the late seventeenth century where he used a cowpox virus to immunize against smallpox infection. Following this, the concept of heterologous immunity and its implications for vaccine development and immunopathogenesis were largely neglected until the late 20th century. However, epidemiological studies involving various vaccinated populations continued to suggest the existence of heterologous immunity. With the availability of modern bioinformatics and immunological tools, heterologous immunity among several pathogens has now been recognized and the immunomodulatory role in protective immunity and immunopathogenesis is being studied extensively. The topic "Heterologous Immunity: Implications and Applications in Vaccines and Immunotherapies" covers current progress in the field of heterologous immunity and is a collection of 13 articles that includes reviews, minireviews and original research.

The review article by Agrawal summarized the mechanism of heterologous immunity and its implications in pathogenesis and infectious disease outcomes. The mechanism of cross-reactive antibody or T cell immunity among related or un-related pathogens lies in the promiscuous nature of the interaction of B cell receptor and T cell receptor for epitope recognition. As a result, exposure to microbiota and various pathogens, and routine vaccination programs induce a considerable pool of cross-reactive T cells as well as antibodies able to respond to a completely different pathogen. The author further describes heterologous immunity among a broad range of pathogens and discusses its plausible role in natural resistance and modulation of the course of infection.

Balz et al. outline the consequences of heterologous immunity in antiviral immunity. In this review, the authors highlight important applications of heterologous immunity in vaccine development. Accordingly, for influenza viruses, they suggest that future vaccine development efforts should be toward a universal vaccine focused on broader T cell responses. The authors further discuss the public health implications of heterologous immunity and suggest that more knowledge on heterologous immunity among various pathogens can provide insight into the repurposing of existing vaccines. They also point out factors that influence the clinical outcomes of heterologous immunity and specifically mention the benefits of measles and vaccinia vaccination in this regard.

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Flaviviruses are among the most common viral infections in tropical and subtropical countries. Heterologous immune responses among Flaviviruses are well-documented. Rathore and St. John review cross-reactive antibody and T cell immunity directed toward Flaviviruses and their role in protection and the development of immunopathologies. The authors also detail rational vaccine design from the viewpoint of cross-reactive immunity.

Malaria, a mosquito-borne disease, is caused by the *Plasmodium* parasite and is among the leading causes of death in endemic areas. In a comprehensive review, Mitran and Yanow describe cross-reactive antibody and T cell immunity among distinct species of *Plasmodium* that have been reported both in human populations and in experimental animal models. The authors define potential antigen targets for cross-species vaccine design in the context of heterologous immunity that would encompass evolutionarily conserved epitopes.

Heterologous immunity is particularly important from an immunopathology standpoint where it can negatively modulate the immune responses against another pathogen. This can occur through the induction of biased non-protective cross-reactive antibodies and T cells. The research article by Tang et al. highlights this phenomenon in a study of infections in mice with mixed-species of malaria parasites. The authors show that co-infection of mice with *Plasmodium yoelii* with either *Plasmodium vinckei* or *Plasmodium chabaudi* increased virulence (100% mortality) compared to mono-infections where mortality was significantly lower (40% with *P. chabaudi* and no mortality with *P. yoelii* or *P. vinckei)*. The authors discuss the role of immune competition/modulation on parasite virulence and the fitness costs of co-infection on transmission to mosquitoes.

Apart from vaccines, heterologous immunity is an attractive approach for the development of immunotherapeutic monoclonal antibodies. Youssef et al. identified a monoclonal IgM antibody against fungus *Candida albicans*' Hyr1 antigen that cross-reacts with the Gram-negative bacteria *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Moreover, this antibody protects mice from lethal bacteremia. This study shows that a single monoclonal antibody can provide cross-kingdom protection against multiple microorganisms.

The T cell receptor has remarkable promiscuity toward its antigen epitopes and this feature is responsible for the generation of cross-reactive T cells. Cross-reactive T cells have several clinical implications for immunopathogenesis, autoimmunity, and alloreactivity and graft rejection. For example, Rowntree et al. published an elegant study where $CD8^+$ T cells specific for different viral epitopes (EBV, CMV, and HIV-1) cross-reacted with HLA-B27 allotypes in a hierarchical manner. This study clearly shows that heterologous immunity has clinical implications wherein antiviral T cells induced against viral pathogens could contribute to adverse outcomes in allogeneic transplantation. It also implies that exposure of a host to multiple viral infections could lead to a more complex allo-reactivity.

The advent of newer T cell-based therapies (such as CAR-T cells) and their widespread clinical uses can be vulnerable to the cross-reactive nature of the T cell receptors. A study by Soon et al. shows that a therapeutic T cell specific to a hepatitis E virus epitope cross-recognized an epitope from an apoptosis-related autoantigen in the host, which could potentially lead to autoimmunity. Most importantly, the authors characterized a molecular signature of a multiple-glycine motif in the CDR3 region of the TCR β chain which may allow greater structural flexibility with a minimum energy threshold required for promiscuity. Therefore, such T cell-based therapies should be evaluated for any off-target cross-reactivity.

Heterologous immunity is also relevant for vaccine development against highly diverse pathogens where cross-strain reactivity or cross-species protection is critically desirable. As an example, current vaccine development efforts against influenza viruses are focused on developing a broadly cross-reactive universal vaccine. These efforts employ modern *in silico* and immunological tools to design cross-reactive, conserved antigen epitopes as well as the use of a suitable adjuvant formulation that favors the induction of antibodies and T cells with broader specificities. Nguyen et al. show that the Pandemic H1N1 vaccine formulated with poly-γ-glutamic acid (PGA)/Alum complex provided cross-protection against heterologous influenza viral strains: A/Puerto Rico/8/34 (H1N1) and A/Hong Kong/1/1968 (H3N2)]. In a similar study, Luo et al. showed that the H7N9 vaccine formulated with STING agonist cGAMP could provide effective cross-protection against H1N1, H3N2, and H9N2 influenza viruses in mice. Finally, Lee et al. showed that mincle and STING-stimulating adjuvant formulated with a foot and mouth disease virus vaccine induced a robust and long-lasting cellular and humoral memory response across diverse species in mice, cattle and pigs. These studies suggest that the use of new, advanced adjuvant formulations will not only induce antibodies and T cells with broader specificities for greatly improved immunity but also overcome disparities in immunogenicity of a vaccine across species.

Heterologous immunity is not only limited to adaptive immunity. Innate immunity triggered by one pathogen or vaccine can protect against an unrelated pathogen. This is often called "trained immunity" and is represented by innate immune cells. Trained immunity arises due to epigenetic reprogramming of innate immune cells such as macrophages upon exposure to infection/vaccine. Covián et al. reviewed BCG vaccine-induced trained innate immunity and its role in cross-protection and heterologous effects.

Furthermore, macrophages play a distinct role in heterologous immunity because they have different functional stages at various points in the course of an infection. This process results in an intrinsic functional imprinting/training of macrophages by the invading pathogen. Connolly and Hussell review several aspects of this training of macrophages and the influence of Type 1 interferons on the alveolar macrophage. The authors also discuss how influenza virus-mediated production of Type 1 interferon alters the functional response of macrophages toward bacterial superinfection.

Collectively, these reviews and original research articles provide a comprehensive overview of the various dimensions of heterologous immunity. They support the outlook that heterologous immunity should be an important aspect in the design, testing and development of vaccines and

immunotherapeutics. In addition, the influence of heterologous immunity should be carefully examined on clinical outcomes of infections and autoimmunity.

AUTHOR CONTRIBUTIONS

SS wrote the manuscript. BA and SY performed critical revision and editing. All authors share equal intellectual contribution to this work and approved it for publication.

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ORIGINAL RESEARCH
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Poly-γ-Glutamic Acid Complexed With Alum Induces Cross-Protective Immunity of Pandemic H1N1 Vaccine

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The use of a good vaccine adjuvant may induce a higher immunogenicity profile of vaccine antigens. Here, we developed a new adjuvant by combining poly-γ-glutamic acid (γ-PGA) with alum (PGA/Alum) and investigated its ability to enhance the immunogenicity and the cross-reactive efficacy of pandemic H1N1 (pH1N1) influenza vaccine antigen. PGA/Alum enhanced antigen delivery to draining lymph nodes and antigen-specific immunogenicity in mice using OVA as a model antigen. It also greatly increased OVA-specific antibody production, cytotoxic T lymphocyte (CTL) activity, and antibody-dependent cellular cytotoxicity (ADCC). These abilities of PGA/Alum improved the protective efficacy of pH1N1 vaccine antigen by increasing hemagglutination-inhibition titers, enhancing ADCC and CTL activity, and speeding viral clearance following homologous viral challenge. Importantly, the cross-protective efficacy of pH1N1 vaccine against heterologous viruses [A/Puerto Rico/8/34 (H1N1) and A/Hong Kong/1/1968 (H3N2)] was significantly enhanced by PGA/Alum, and cross-reactive ADCC and CTL activities were observed. Together, our results strongly suggest that PGA/Alum may be a promising vaccine adjuvant for preventing influenza and other infectious diseases.

Keywords: vaccine adjuvant, influenza virus, efficacy, cross-protection, antibody-dependent cellular cytotoxicity, cytotoxic T lymphocyte activity

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INTRODUCTION

Traditional vaccines are composed of killed or attenuated viruses or bacteria and have several drawbacks, including safety concerns, the need for complicated culture of the infectious agents, and the low yields of their manufacturing processes (1, 2). To solve these problems, researchers have developed new types of vaccine such as subunit recombinant vaccines and DNA vaccines (3). Unfortunately, most of these vaccines are unable to generate sufficient antigen-specific immunogenicity to effectively prevent infectious diseases. In particular, influenza continues to occur as a seasonal epidemic and in sporadic pandemics with considerable morbidity and mortality worldwide, largely because we lack an effective vaccine capable of inducing broad cross-protection against newly emerging influenza viruses that underwent antigenic drift and shift (4). The future development of effective influenza vaccines has been proposed to hinge on the use of adjuvants that improve the immunogenicity and cross-reactive immunity of vaccine antigens (5, 6).

Despite extensive research, relatively few adjuvants have been licensed for use with human vaccines. These adjuvants included aluminum salts (alum) and emulsions (e.g., MF59) (6, 7). Since the 1920s, alum has been used as a vaccine adjuvant for a wide range of vaccines in the US and Europe. It can trigger robust humoral immune responses (i.e., antibody production), but does little to enhance the antibody-dependent cellular cytotoxicity (ADCC) and cytotoxic T lymphocyte (CTL) activities that are critical for the protection against various pathogens, including viruses and intracellular pathogens, and cross-reactivity against heterologous influenza viruses (8–11). Regarding MF59, its adjuvanted influenza vaccine did not show any significant difference in the cross-reactivity compared to the unadjuvanted vaccine (12), and MF59 itself has been associated with adverse effects, including injection site pain and the induction of inflammatory arthritis (13).

The current approach for developing vaccine adjuvants is based on our knowledge of the innate immune responses that initiate adaptive immune responses. Some adjuvants include agonists that can enhance innate immune responses through pattern recognition receptors, such as toll-like receptors (TLRs) (14, 15). Poly-γ-glutamic acid (γ-PGA) is a natural, biodegradable, and edible biopolymer composed of repeating units of both D- and L-glutamic acids combined via γ-amide linkages that is secreted naturally by the *Bacillus subtilis sups. Chungkookjang* commonly found in Korean traditional soybean paste, chungkookjang (16). We previously reported that γ-PGA induces TLR4-mediated innate immune responses and robustly provokes Th1 immune responses to enhance CTL activity (17). Taking advantage of the safety and potential immunostimulatory properties of γ-PGA, we developed a vaccine adjuvant by combining γ-PGA with alum (PGA/Alum) to resolve the limitations of currently licensed vaccine adjuvants. We investigated the physiochemical properties, efficacy, and action mechanisms of PGA/Alum using the model antigen, ovalbumin (OVA), *in vivo* and *in vitro*. We then evaluated the adjuvant efficacy of PGA/Alum in improving pandemic H1N1 (pH1N1) influenza vaccine antigen-specific cellular immune responses, antibody (Ab) production, and cross-reactivity against heterologous influenza A viruses. Our results demonstrate that PGA/Alum increased dendritic cell (DC) activation and antigen trafficking, thereby enhancing adaptive immune responses, particularly antigen-specific CTL activity and ADCC. Furthermore, the protective and cross-reactive efficacies of pH1N1 influenza vaccine were substantially improved by PGA/Alum, which conferred cross-protection accompanied with cross-reactive ADCC and CTL activities. Together, our results strongly suggest that PGA/Alum may be a promising vaccine adjuvant for prevention of influenza-related and other infectious diseases.

MATERIALS AND METHODS

Mice

Six- to eight-week-old female C57BL/6 mice (Orient Bio) were housed in a specific pathogen-free (SPF) facility in the Korea Research Institute of Bioscience and Biotechnology (KRIBB). Handling of mice and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the KRIBB (KRIBB-AEC-17013 and KRIBB-AEC-17162) and were performed according to the Guidelines for Animal Experiments of the KRIBB.

Cells

Bone marrow-derived DCs (BMDCs) were generated and maintained in RPMI 1640 (Gibco) that contained 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco), as previously described (18). B16F10 and MDCK cells were purchased from ATCC and maintained in DMEM (Gibco) that contained 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. B16mOVA cells (B16F10 cells expressing membrane-bound OVA) were kindly provided by Dr. David J. Mooney (Harvard University, USA) and were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 μg/ml puromycin dihydrochloride (Millipore).

Preparation of PGA/Alum

PGA/Alum was prepared by combining γ-PGA (BioLeaders) and Imject alum (Thermo Fisher) in a 0.9% saline solution. Briefly, 1 mg/ml alum solution, pH 6.5 (adjusted by HCl) was added drop-wise into 1 mg/ml γ-PGA solution, pH 6.8 (adjusted by ammonia solution) (v:v = 1:1) with constant stirring at 70 × *g*. The resultant PGA/Alum was collected by centrifugation at 15,000 × *g* for 30 min at 4°C, re-suspended in a 0.9% saline solution, and stored at 4°C prior to use.

Preparation of Viruses

The influenza viruses A/California/04/09 (pH1N1), A/Puerto Rico/8/34 (H1N1), and A/Hong Kong/1/68 [H3N2 (a reassortant H3N2 virus carrying the HA and NA genes from A/Hong Kong/1/68 and internal genes from A/Puerto Rico/8/34)], were grown in 9 to 10-day-old SPF embryonated chicken eggs (Orient Bio) for 48 h at 37°C. The viruses were harvested from the allantoic fluids by centrifugation at 3,500 × *g* for 10 min at 4°C and filtration through 0.45 μm pore-size membrane filter (Millipore) and then stored at −80°C until use. All viral experiments were performed under biosafety level 2+ (BSL2+) conditions.

Dynamic Light Scattering (DLS)

The hydrodynamic diameter and polydispersity index of alum and PGA/Alum were measured using a particle-size analyzer (ELS-Z; Otsuka Inc.). Zeta-potential values were measured with a Zeta-sizer (Nano ZS; Malvern Instruments Ltd.).

Scanning Electron Microscopy (SEM)

The morphologies of alum and PGA/Alum were observed using a field-emission scanning electron microscope (FE-SEM, Quanta 250 FEG). Briefly, alum and PGA/Alum (100 μg/ml, 1 ml) were dispersed in autoclaved saline buffer, dropped and dried on a silicon wafer, coated with gold for 60 s using a Polaron SC7640 sputter coater (Quorum Technologies Ltd.) and then subjected to SEM.

Transmission Electron Microscopy (TEM)

TEM images of alum and PGA/Alum were obtained using a field-emission transmission electron microscope (FE-TEM; JEOL Ltd.). For visualization, alum and PGA/Alum (100 μg/ml) solutions were dropped and dried on a formvar- and carbon-coated copper grid (Ted Pella, Inc).

Fourier Transform Infrared (FT-IR) Spectroscopy

To analyze the chemical structure of PGA/Alum, we performed FT-IR analysis using FT-IR spectroscopy (Alpha-T; Bruker Optics). Alum and PGA/Alum solutions (1 mg/ml in saline buffer) were centrifuged at 10,000 × *g* for 5 min and re-dispersed in distilled water. After all solutions were dried under a vacuum for 2 days, the powder was mixed with potassium bromide (KBr). FT-IR signals were obtained by scanning from 500 to 4,000 cm^{-1} with a scan resolution of 4 cm^{-1}.

Determination of OVA Antigen Loading

PGA/Alum and alum (10 mg/ml each) were mixed overnight with OVA antigen (3 mg/ml; Sigma-Aldrich) at 4°C with constant rotation at 15 × *g*. The resulting PGA/Alum-OVA complexes were centrifuged at 14,000 × *g* for 15 min, and the supernatants were collected for the analysis of unloaded OVA. The amount of loaded OVA was calculated based on the amount of unloaded OVA in the supernatants, as assessed via BCA protein assay (Pierce).

Cell Viability Assay

Immature BMDCs were generated from bone marrow cells of C57BL/6 mice and stimulated with alum, γ-PGA, or PGA/Alum at 25, 50, 100, or 200 μg/ml for 24 h. The cell viability was measured using the Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's instructions.

In vitro Activation and Antigen Uptake/Processing of BMDCs

To investigate BMDC activation, we stimulated the cells with 100 μg/ml alum, 100 μg/ml γ-PGA, or 200 μg/ml PGA/Alum for 24 h at 37°C. To examine the antigen uptake and processing of BMDCs, we incubated the cells with either 5 μg/ml FITC-OVA (Thermo Fisher) or 5 μg/ml DQ-OVA (a self-quenching conjugate of OVA that exhibits bright green fluorescence upon proteolytic degradation; Thermo Fisher) mixed with or without alum, γ-PGA, or PGA/Alum for 1 or 5 h at 37°C, respectively.

ELISA

The cytokine levels in the culture supernatants were measured using ELISA kits (BD Bioscience) according to the manufacturer's instructions. To determine the levels of antigen-specific IgG Ab in the sera of immunized mice, ELISA plates (Nunc) were coated overnight with 1 μg/ml OVA protein or 0.5 μg/ml influenza vaccine antigen (A/California/7/2009 NYMC X-179A H1N1; provided by Mogam Biotechnology Research Institute) in carbonate solution, pH 9.5, at 4°C. ELISA was performed as previously described (18, 19).

Flow Cytometry

All cells were blocked with anti-CD16/CD32 monoclonal Ab (mAb) and stained with the subsequently described fluorochrome-conjugated mAbs. The mAbs were purchased from BD Biosciences, BioLegend, or eBioscience. To measure DC activation, cells were stained with PE-conjugated mAbs against mouse CD40, CD80, CD86, MHC class II molecules or isotype-matched control mAbs. To examine the proportions of DCs located at the injection sites and draining lymph nodes (dLNs), C57BL/6 mice were intramuscularly (i.m.) immunized with 5 μg Alexa Fluor 647-conjugated OVA mixed with or without 800 μg PGA/Alum, and cells were obtained after 3, 6, 12, and 48 h post-immunization. In other experiments, C57BL/6 mice were i.m. immunized with 5 μg Alexa Fluor 647-conjugated OVA alone or mixed with 400 μg alum, 400 μg γ-PGA, or 800 μg PGA/Alum, and cells were obtained after 24 h post-immunization. The cells were stained with APC eFluor 780-conjugated anti-CD11c and Alexa Fluor 488-conjugated anti-MHC class II mAbs. To measure frequency of $OVA_{257-264}$ tetramer-positive $CD8^+$ T cells, splenocytes were obtained from the immunized mice and blocked with clear back (Fc receptor blocking reagent; MBL), stained with PE-conjugated H-$2K^b$ OVA tetramer and FITC-conjugated CD8 mAbs (MBL). All stained cells were acquired on FACSCalibur or FACSVerse flow cytometers (BD), and the data were analyzed using FlowJo software (Tree Star). Fluorescence compensation was optimized using cells individually labeled with each fluorochrome-conjugated mAb. Data were obtained from the live population based on cell size- and morphology-based gating, which was used to eliminate cell debris and dead cells.

In vivo Fluorescence Imaging

To visualize the migration of antigen to the dLNs, a fluorescent dye-labeled antigen was prepared by conjugating 1 mg OVA protein with 0.1 mg IRDye800CW fluorescent dye using an IRDye800CW protein labeling kit (LI-COR Bioscience). The concentration of the resulting IRDye800CW-labeled OVA (OVA-IR800) was determined using a BCA protein assay. Hair on the left forepaw and the dorsal skin of C57BL/6 mice were removed by applying depilatory creams (VEET Hair Removal Cream; Reckitt Benckiser Japan) for efficient signal transmission. The mice were anesthetized with 3% isoflurane and intradermally administered 25 μg OVA-IR800 alone or mixed with 400 μg alum, 400 μg γ-PGA, or 800 μg PGA/Alum into the forepaw pad. At 1, 3, 6, 24, and 48 h post-injection, *in vivo* near-infrared (NIR) fluorescent signals from the anesthetized mice were acquired using the *in vivo* Imaging System (IVIS Lumina II; Xenogen Corp.) with excitation at 780 nm and emission at 831 nm at a 0.02 s exposure time. The fluorescent signals of OVA-IR800 in the axillary lymph node were quantitatively analyzed using image analysis ImageJ software (NIH).

Immunizations and Viral Challenge

Animals were randomly distributed to groups of 3–6 mice. C57BL/6 mice were i.m. immunized with 10 μg OVA in the presence or absence of 400 μg/ml γ-PGA, 400 μg/ml alum, or 800 μg/ml PGA/Alum on days 0, 14, and 28. Spleens, bone marrow cells, and sera were collected on days 14 and

180 after the last immunization. In a separate experiment, mice were i.m. immunized with the pH1N1 split vaccine antigen (A/California/7/2009 NYMC X-179A H1N1), which contained 0.05 μg (for homosubtypic protection) or 0.5 μg (for heterosubtypic protection) hemagglutinin (HA) plus 400 μg γ-PGA, 400 μg alum, or 800 μg PGA/Alum on days 0 and 14. Spleens and sera were collected on day 14 after the last vaccination. Two weeks after the last vaccination, the mice were intranasally challenged with a lethal dose (LD) of influenza viruses, including 50 LD_{50} (equivalent to 30,000 $TCID_{50}$ or 15,000 PFU) A/California/04/09 (pH1N1), 10 LD_{50} (equivalent to 20 $TCID_{50}$ or 200 PFU) A/Puerto Rico/8/34 (H1N1), or 10 LD_{50} (equivalent to 600 $TCID_{50}$ or 300 PFU) H3N2 viruses.

The body weight and survival were monitored for 14 days after the viral challenge. Mice that lost >20% (for homosubtypic viral challenge) or 25% (for heterosubtypic viral challenge) of their body weight were considered to have reached the experimental end point and were sacrificed.

Antibody-Dependent NK Cell Activation

To examine the ability of the sera Abs of immunized mice to activate NK cells, we coated ELISA plates (Nunc) overnight at 4°C with 10 μg/ml OVA protein or 6 μg/ml influenza HA antigen that contained the stalk domain. The plates were washed with PBS, incubated with heat-inactivated sera (1 h at 56°C) for 2 h at 37°C, and washed with PBS to remove unbound serum Abs. NK cells were isolated from the splenocytes of unimmunized C57BL/6 mice using a NK cell isolation kit (Miltenyi Biotec). The isolated NK cells were dispensed to the ELISA plates (1×10^5 cells/well) and incubated in the presence of PE-conjugated anti-CD107a mAb, 5 μg/ml brefeldin A, and 5 μg/ml monensin for 5 h at 37°C. Finally, the NK cells were harvested, fixed, permeabilized, and intracellularly stained with APC-conjugated anti-IFN-γ using a Cytofix/Cytoperm kit (BD Bioscience).

ADCC Assay

For experiments using the sera of mice immunized with OVA antigen, B16mOVA cells (target cells) were plated to 96-well U-bottom plates at 8×10^3 cells/well. B16F10 cells were used as a negative control. For experiments using the sera of mice immunized with influenza vaccine antigen, MDCK cells were infected with A/California/04/09 (pH1N1), A/Puerto Rico/8/34 (H1N1), or H3N2 (multiplicity of infection = 1) in serum-free DMEM that contained 100 U/ml penicillin and 100 mg/ml streptomycin for 12 h at 37°C. The virus-infected MDCK cells (target cells) were harvested and plated to 96-well U-bottom

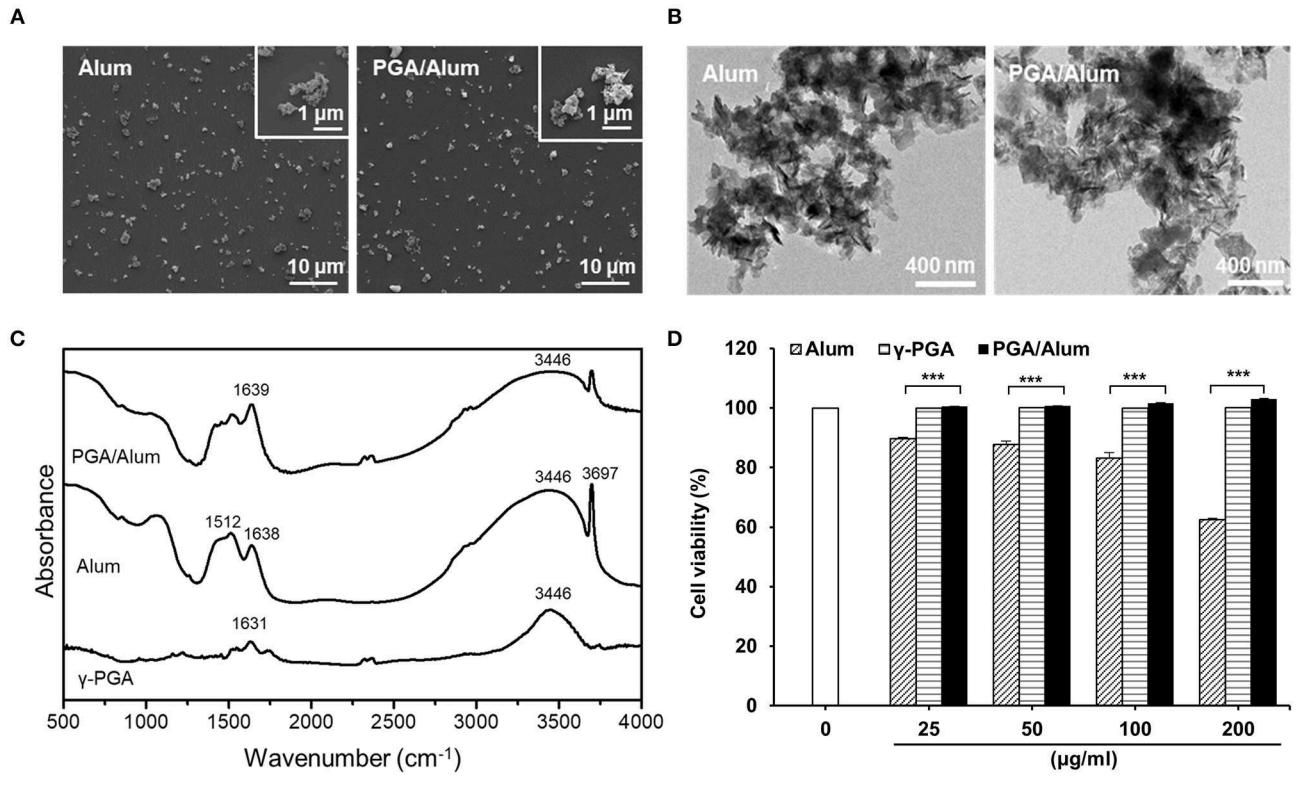

FIGURE 1 | The physiochemical characterization of PGA/Alum. **(A)** SEM and **(B)** TEM images of alum and PGA/Alum (inset images are highly magnified). **(C)** FT-IR spectra of γ-PGA, alum, and PGA/Alum. **(D)** Cytotoxicity of PGA/Alum was evaluated by measuring cell viability of BMDCs exposed to various concentrations of alum, γ-PGA, and PGA/Alum for 24 h. The data are representative of three independent experiments. Statistically significant differences were analyzed via ANOVA/Bonferroni. ***$P < 0.001$.

TABLE 1 | Physiochemical characterization and OVA-loaded efficiencies of PGA/Alum.

Sample	Particle size (diameter nm ± SD)	Polydispersity index (PDI)	Zeta potential (mV)	OVA-loaded (mg/ml)
Alum	1,066.2 ± 32.01	0.31 ± 0.03	−7.08 ± 0.91	1.603 ± 0.04
PGA/Alum	1,294.67 ± 13.32	0.37 ± 0.03	−28.10 ± 1.49	2.144 ± 0.08

plates at 8×10^3 cells/well. The target cells were mixed with both heat-inactivated serum samples (56°C, 1 h) and naïve NK cells and then incubated for 4 h at 37°C. Cytotoxicity was assessed by detection of lactate dehydrogenase (LDH) in culture supernatants using a CytoTox 96 Non-radioactive cytotoxicity assay (Promega).

Enzyme-Linked Immunospot (ELISPOT) Assay

The frequencies of antigen-specific IFN-γ-producing cells were evaluated using a mouse ELISPOT kit (BD Bioscience), as previously described (18, 19). Briefly, splenocytes were obtained from immunized mice and plated at 5×10^5 cells/well onto purified anti-IFN-γ-coated ELISPOT plates. The cells were stimulated with $OVA_{257-264}$ peptide (0.5 μg/well; Anaspec) or UV-inactivated viruses, including 500 $TCID_{50}$/well of A/California/04/09 (pH1N1), 2,000 $TCID_{50}$/well of A/Puerto Rico/8/34 (H1N1), or 2,000 $TCID_{50}$/well of H3N2 viruses for 3 days. The spot-forming units (SFUs) of IFN-γ-producing cells were counted using an ELISPOT plate reader (Cellular Technology Ltd).

Hemagglutination-Inhibition (HI) Assay

The serum HI titers against the A/California/04/09 (pH1N1) were determined as previously described (18). HI titers were recorded as the reciprocals of the highest serum dilution at which hemagglutination was prevented.

Virus Titration in Lungs

On days 3 and 7 post-challenge, mouse lung samples were homogenized in MEM that contained 0.2% BSA at 1 g of lung/ml. The homogenates were centrifuged at 15,000 × *g* for 10 min at 4°C to remove cell debris, and the supernatants were used for the assay. The viral titers were calculated according to the method of Reed and Muench (20) and expressed as $\log_{10}$ $TCID_{50}$/ml.

Statistical Analysis

Data are presented as the means ± standard deviations (SDs) and represent at least three independent experiments. Significant differences between two groups were assessed using the two-tailed Student's *t*-test, and differences among multiple groups were assessed using one-way ANOVA followed by Bonferroni's correction (ANOVA/Bonferroni). The log-rank test was used to analyze survival between two groups. *P* values <0.05 were considered to be statistically significant. All analyses were performed using GraphPad PRISM software (GraphPad).

RESULTS

PGA/Alum Complex Shows a High Antigen-Loading Capacity With No Cytotoxicity

To develop a potential adjuvant for future clinical applications, we fabricated PGA/Alum by combining γ-PGA and alum, which is composed of aluminum hydroxide (AH) and magnesium hydroxide (MH). The size and morphology of the fabricated PGA/Alum were measured using dynamic light scattering, TEM, and SEM. Similar to alum, PGA/Alum exhibited an irregular morphology with an average diameter of 1–2 μm (**Figures 1A,B**), which is consistent with the morphology of alum in a previous report (21). As shown in **Table 1**, the hydrodynamic diameter of PGA/Alum (1,294.67 ± 13.32 nm) was larger than that of alum (1,066.2 ± 32.01 nm). The polydispersity index was 0.37 ± 0.03 for PGA/Alum and 0.31 ± 0.03 for alum, and was shown in the mid-range of the index value from 0.08 to 0.7 (22). The zeta-potential value of alum was −7.08 ± 0.91 mV, while the value of the PGA/Alum complex was −28.10 ± 1.49 mV, which suggests that γ-PGA was successfully conjugated to alum. To confirm the chemical structures of γ-PGA, alum, and PGA/Alum, we used FT-IR analysis (**Figure 1C**). In the γ-PGA spectrum, a broad peak at ∼3,446 cm^{-1} was attributed to N-H and O-H stretching, while that at 1,631 cm^{-1} was attributed to the C=O stretching of the carbonyl, as previously reported for γ-PGA (23). According to the manufacturer's description, the utilized alum is composed of AH and MH with inactive stabilizers. In the alum spectrum, the peaks at 3,697 and 3,446 cm^{-1} were attributed to the hydroxyl groups of MH and AH, respectively, and those at 1,638 and 1,512 cm^{-1} were attributed to vibrations of the Mg-OH and OH bonds, respectively, in the crystal structure (24–26). The spectrum of PGA/Alum showed distinct peaks that corresponded to alum and γ-PGA at 3,446 and 1,639 cm^{-1}, respectively. Based on the results of the FT-IR analysis, we confirmed that γ-PGA and alum successfully formed a complex. We further examined the cytotoxicity of PGA/Alum using BMDCs exposed to alum, γ-PGA, or PGA/Alum for 24 h. As shown in **Figure 1D**, the treatment of γ-PGA or PGA/Alum did not affect the cell viability, whereas alum dose-dependently decreased the cell viability as previously reported (21). Additionally, immunofluorescent microscopic analysis showed that PGA/Alum was efficiently taken up by BMDCs *in vitro* (**Figure S1A**). Production of IL-6 and IL-1β rapidly and transiently increased by PGA/Alum in the injection sites by 6 h post-injection but declined almost to basal levels by 24 h post-injection (**Figure S1B**). We further confirmed the safety of PGA/Alum by measuring body temperature and levels of inflammatory cytokines (IL-6 and TNF-α) in the sera of the vaccinated mice at 6, 24, 48, 72, 96, and 120 h

post-vaccination. The PGA/Alum-vaccine group showed no significant change of body temperature compared with PBS or vaccine groups (**Figure S2A**). The level of serum IL-6 was higher in the PGA/Alum-vaccine and vaccine groups than PBS group at 6 h post-vaccination, but it maintained a basal level at 24 h post-injection. Serum TNF-α level was not changed in PGA/Alum-vaccine, vaccine, and PBS groups (**Figure S2B**). These results support the safety of PGA/Alum after vaccination. To investigate the antigen encapsulation efficiency, we mixed PGA/Alum with OVA as a model protein antigen and used the BCA assay to measure the amount of loaded OVA. Our results indicated that PGA/Alum loaded significantly more OVA (2.144 ± 0.08 mg/ml) than alum (1.603 ± 0.04 mg/ml) (**Table 1**). Taken together, our results indicate that the PGA/Alum complex successfully form under the utilized conditions and it shows a high antigen-loading capacity without cytotoxicity.

PGA/Alum Significantly Enhances the Activation and Antigen Presentation of DCs *in vitro*

Because DCs are professional antigen-presenting cells (APCs) that are responsible for the initiation of adaptive immunity (27), we examined the effect of PGA/Alum on DC activation. After immature BMDCs were prepared and stimulated with alum, γ-PGA, or PGA/Alum for 24 h, the levels of pro-inflammatory cytokines and various costimulatory molecules were analyzed via ELISA and flow cytometry, respectively. As shown in **Figure 2A**, the mean fluorescence intensities (MFIs) of costimulatory molecules (CD40, CD80, and CD86) and MHC class II molecules were substantially increased on the PGA/Alum-exposed BMDCs compared to those treated with alum or γ-PGA. Similarly, PGA/Alum-treated BMDCs produced higher levels of cytokines (TNF-α, IL-6, IL-1β, and IL-12) than those stimulated with alum or γ-PGA alone ($P < 0.01$) (**Figure 2B**). To investigate the antigen uptake and degradation abilities of these cells, we incubated BMDCs with FITC-OVA or DQ-OVA, which is a self-quenching dye that emits green fluorescence upon the degradation of OVA. Flow cytometry showed that the MFIs of FITC-OVA or DQ-OVA were significantly higher in the PGA/Alum-treated BMDCs than in those treated with PBS, alum, or γ-PGA (**Figure 2C**), indicating that PGA/Alum substantially increases the antigen-uptake ability of DCs.

Because γ-PGA has been reported to activate DCs via TLR4 signaling (17), we prepared BMDCs from TLR4-defective C3H/HeJ mice and used them to examine whether PGA/Alum-induced DC activation is mediated by TLR4. We observed a significantly lower level of TNF-α in BMDCs from TLR4-defective C3H/HeJ mice than from wild-type C3H/HeN mice following PGA/Alum treatment (**Figure S3A**). Furthermore, PGA/Alum induced little IκBα phosphorylation in TLR4-defective BMDCs, whereas the phosphorylation in wild-type BMDCs was dose-dependently increased by PGA/Alum (**Figure S3B**). Taken together, these results indicate that PGA/Alum activates DCs through NF-κB signaling via TLR4.

PGA/Alum Substantially Increases Antigen Trafficking and Migration of Antigen-Loaded DCs From Injection Sites to Draining Lymph Nodes

The recruitment of DCs to the antigen injection site and the migration of antigen-loaded DCs to dLNs are vital steps in the induction of adaptive immunity and are important to the efficacy of a vaccine. Accordingly, we used Alexa Fluor 647-conjugated OVA (Fluor-OVA) to examine the effect of PGA/Alum on the recruitment of DCs and the migration of antigen-loaded DCs. Mice were i.m. injected with Fluor-OVA mixed with or without PGA/Alum, and flow cytometry was used to enumerate the DCs in injected muscle tissues and dLNs. Compared with the Fluor-OVA group, the number of DCs ($CD11c^+MHC\text{-}II^+$) was significantly increased in the muscle tissues of the PGA/Alum-Fluor-OVA group at 6 and 24 h post-injection (**Figure 3A**). Notably, we observed ∼2-fold more DCs in the muscle tissues of the PGA/Alum-Fluor-OVA group (13.3 ± 2.2 × 10^3 cells/muscle) than in the Fluor-OVA group (5.5 ± 0.7 × 10^3 cells/muscle) at 24 h post-injection. Importantly, the number of antigen-loaded DCs ($Fluor\text{-}OVA^+CD11c^+MHC\text{-}II^+$) was increased in the dLNs of the PGA/Alum-Fluor-OVA group compared to the Fluor-OVA group, particularly at 6 h post-injection (12.5 ± 0.1 × 10^3 cells/LN for the PGA/Alum-Fluor-OVA group and 7.1 ± 2.5 × 10^3 cells/LN for the Fluor-OVA group) ($P < 0.05$) (**Figure 3B**). The fluorescent intensity of Fluor-OVA and the accumulation of $CD11c^+MHC\text{-}II^+$ cells increased in both the injected muscle tissues and dLNs of the PGA/Alum-Fluor-OVA group at 6 h post-injection (**Figure S4A**). We also observed increased numbers of neutrophils and monocytes in the injected muscles of the PGA/Alum-Fluor-OVA group compared with the Fluor-OVA group at 3, 6, and 24 h post-injection (**Figure S4B**). Compared to other groups, the PGA/Alum-Fluor-OVA group had significantly higher DC numbers in injected muscle tissues and dLNs (**Figure 3C**) and higher antigen-loaded DCs numbers in dLNs (**Figure 3D**) at 24 h post-injection.

To assess the antigen delivery activity of PGA/Alum, we performed *in vivo* imaging of the antigen trafficking by PGA/Alum from the injection site to the dLNs using OVA with an NIR fluorescence imaging system. IRDye800-labeled OVA (OVA-IR800) alone or combined with alum, γ-PGA, or PGA/Alum were subcutaneously (s.c.) injected into the right forepaw pad of C57BL/6 mice, and *in vivo* fluorescent signals were observed at 1, 3, 6, 24, and 48 h post-injection. As shown in **Figure 3E**, the fluorescent intensities were significantly higher in the dLNs of the mice treated with PGA/Alum-mixed OVA-IR800 than in those of the mice exposed to OVA-IR800 alone or mixed with alum or γ-PGA at 6, 24, and 48 h post-injection. Notably, the sustained fluorescent signal was observed in the dLNs of the PGA/Alum-mixed OVA-IR800-treated mice until 48 h post-administration, but not in other groups, which implies that PGA/Alum acts as an efficient antigen carrier.

As chemokines modulate the migration of immune cells to dLNs (28–30), levels of various chemokines (MIP-1α, MIP-1β,

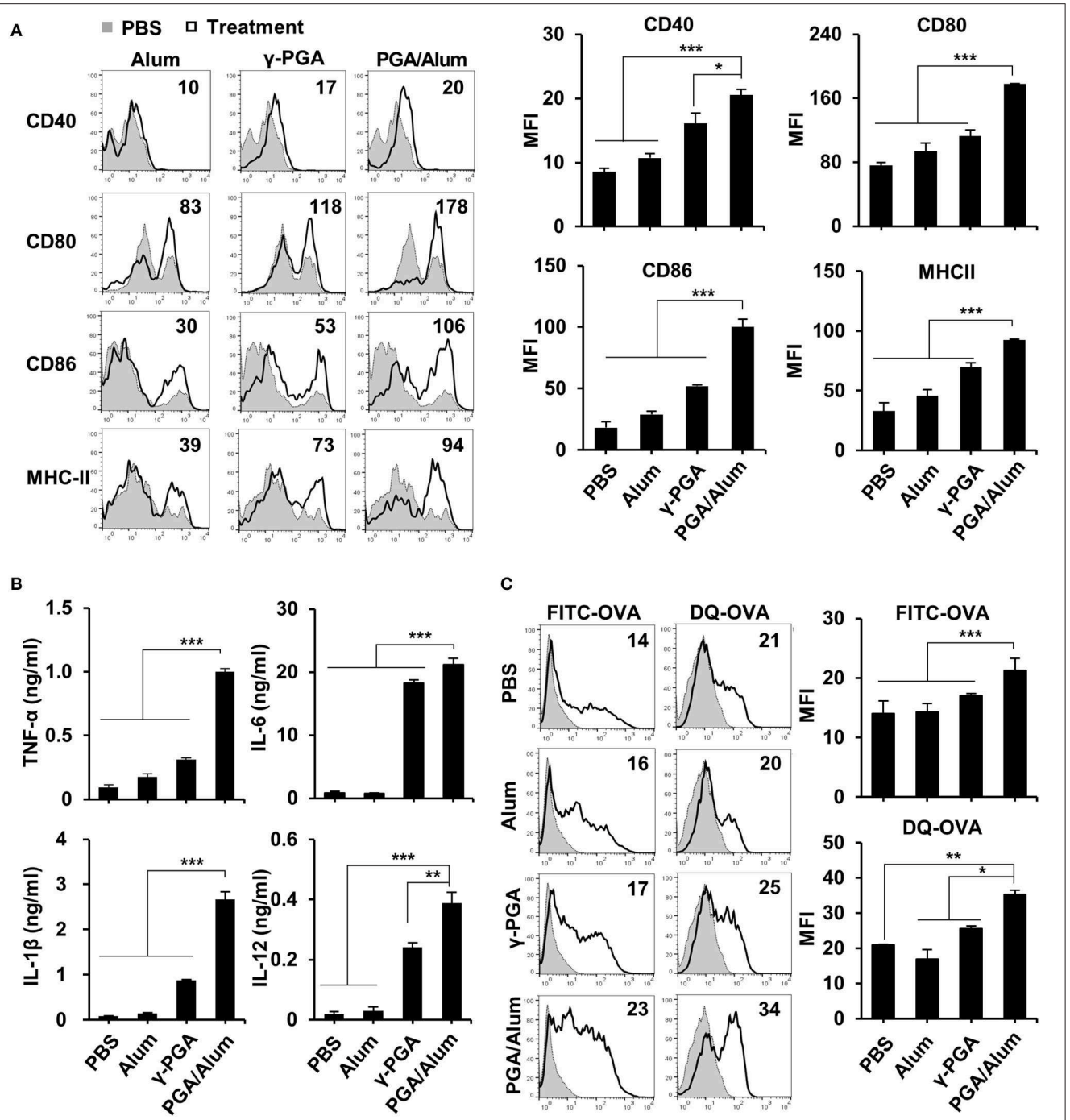

FIGURE 2 | PGA/Alum significantly induces activation and antigen presentation of DCs. Immature BMDCs were stimulated with alum, γ-PGA, and PGA/Alum for 24 h. **(A)** Expression levels of CD40, CD80, CD86, and MHC-II were analyzed by flow cytometry, and **(B)** the levels of cytokines in the culture supernatants were determined by ELISA. **(C)** Immature BMDCs were incubated with either FITC-OVA or DQ-OVA plus PBS or alum, γ-PGA, and PGA/Alum for 1 or 5 h, respectively. Fluorescent intensity was measured via flow cytometry. The numbers in the histograms indicate MFI values. The data are representative of three independent experiments. Statistically significant differences were analyzed via ANOVA/Bonferroni; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

and MCP-1) were substantially enhanced in the homogenates from the muscle tissues of the PGA/Alum-OVA group, but not in those of the OVA group ($P < 0.001$) (**Figure S5A**). Increased chemokine levels were also observed in the dLN homogenates of the PGA/Alum-OVA group, but not in those of the OVA group. We additionally examined the expression of CCR7 responsible for migration of DCs from the antigen exposure site to dLNs (31). As expected, the expression of CCR7 was nearly 3-fold higher on

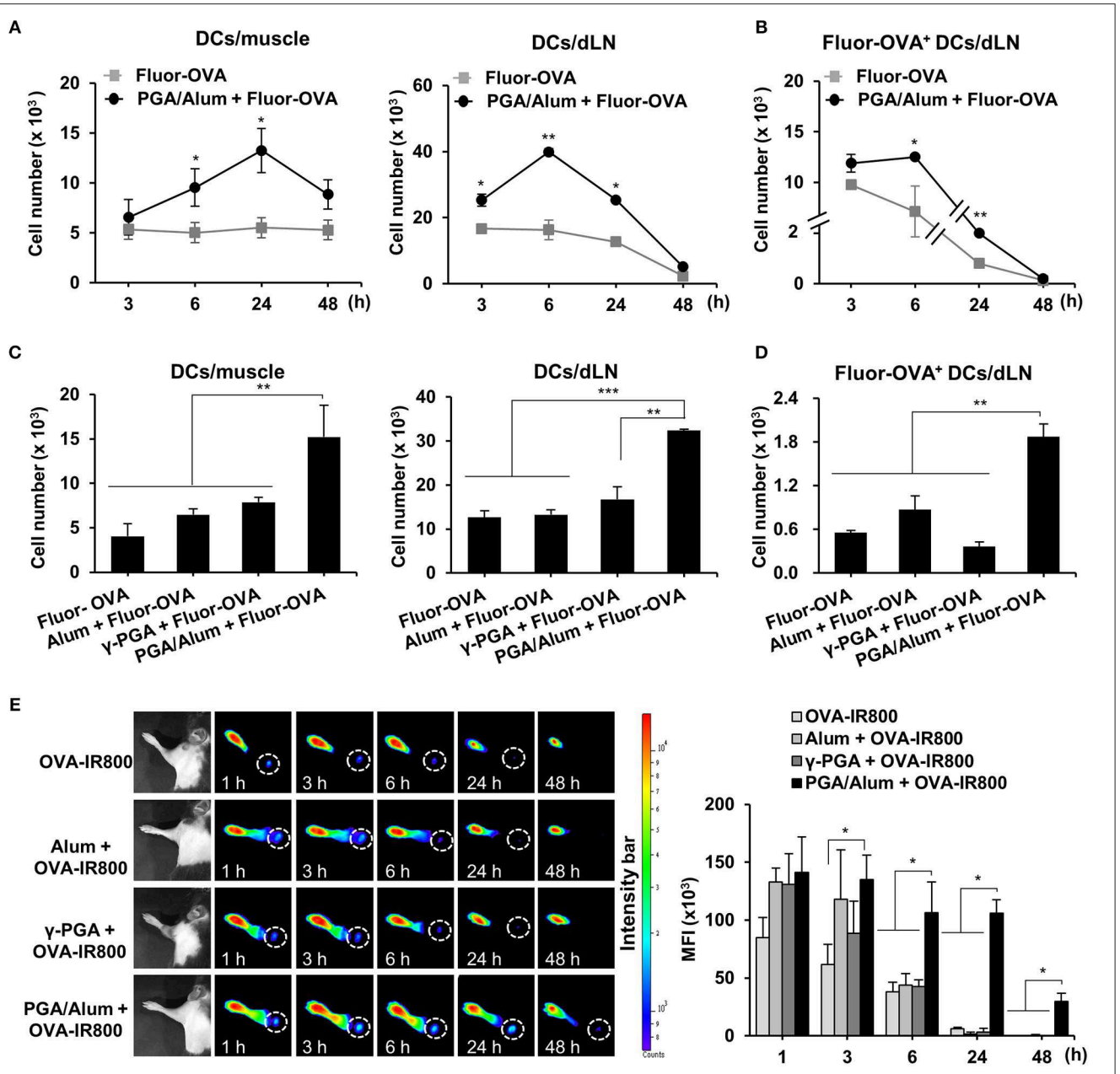

FIGURE 3 | PGA/Alum efficiently increases antigen trafficking from the injection site to dLNs. **(A,B)** C57BL/6 mice ($n = 3$ per group) were i.m. immunized with 5 μg Fluor-OVA mixed with 800 μg PGA/Alum. At 3, 6, 24, and 48 h post-immunization, the number of DCs (gated as $CD11c^+MHC\text{-}II^+$ cells) per injected muscle and dLN **(A)** and Fluor-OVA-loaded DCs (gated as $Fluor\text{-}OVA^+CD11c^+MHC\text{-}II^+$ cells) per dLN **(B)** were analyzed via flow cytometry. **(C,D)** C57BL/6 mice ($n = 3$ per group) were i.m. immunized with 5 μg Alexa Fluor 647-conjugated OVA alone or combined with 400 μg alum, 400 μg γ-PGA, or 800 μg PGA/Alum. At 24 h post-immunization, the number of DCs per injected muscle and dLN **(C)** and Fluor-OVA-loaded DCs per dLN **(D)** were analyzed via flow cytometry. **(E)** Mice ($n = 3$ per group) were s.c. injected into the right forepaw pad with 25 μg OVA-IR800 alone or combined with 400 μg alum, 400 μg γ-PGA, or 800 μg PGA/Alum. At the indicated time points, *in vivo* NIR fluorescence signals were acquired using IVIS. Fluorescent intensities of each region of interest were quantitatively measured using ImageJ software (circle: axillary lymph node). The data are representative of three independent experiments. Statistically significant differences were identified via *t*-test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N.D, not-detected.

the PGA/Alum-exposed DCs than on those exposed to alum or γ-PGA (**Figure S5B**). Also, the mice administered PGA/Alum-mixed Fluor-OVA had significantly higher expression of CCR7 on the DCs in the dLNs than other groups (**Figure S5C**). Collectively, these results indicate that PGA/Alum enhances the recruitment of DCs to injection sites and the migration of antigen-loaded DCs to dLNs through increases of chemokine production and CCR7 expression.

PGA/Alum Enhances OVA-Specific Humoral and Cellular Immune Responses

To evaluate the effect of PGA/Alum on antigen-specific humoral and cellular immunity, we i.m. injected mice with OVA mixed with alum (alum-OVA), γ-PGA (γ-PGA-OVA), or PGA/Alum (PGA/Alum-OVA) on days 0, 14, and 28. Two weeks after the last immunization, ELISA was employed to measure the levels of OVA-specific Abs in the sera of the immunized mice. Our results showed that the levels of IgG, IgG1, and IgG2b significantly increased in the sera of the PGA/Alum-OVA group compared with those of the OVA, alum-OVA, and γ-PGA-OVA groups ($P < 0.001$) (**Figure 4A**; **Figure S6**). This finding indicates that PGA/Alum increases antigen-specific humoral immunity.

We subsequently examined the ADCC of natural killer (NK) cells, which is crucial for eliminating Ab-bound target cells (e.g., virus-infected cells) (32, 33). To investigate the effect of PGA/Alum on the ADCC activity of NK cells, we examined the CD107a expression and the IFN-γ production of NK cells, as well as target cell cytolysis using B16mOVA cells (OVA-expressed B16F10 cells). The activation of NK cells was robustly enhanced by co-culture with a mixture of B16mOVA cells and the sera of the PGA/Alum-OVA group (5.5 ± 1.2% for IFN-γ^+CD107a$^+$ and 39.6 ± 4.6% for CD107a$^+$) compared with the OVA (1.2 ± 0.1% for IFN-γ^+CD107a$^+$ and 25.9 ± 1.6% for CD107a$^+$), γ-PGA-OVA (1.2 ± 0.3% for IFN-γ^+CD107a$^+$ and 25.8 ± 2.4% for CD107a$^+$), and alum-OVA (2.1 ± 0.1% for IFN-γ^+CD107a$^+$ and 27.3 ± 1.9% for CD107a$^+$) groups (**Figure 4B**; **Figure S7A**). The percentage of cytotoxicity was also higher in the PGA/Alum-OVA group (60 ± 5%) than in the γ-PGA-OVA (50 ± 4%), alum-OVA (50 ± 8%), or OVA (41 ± 3%) groups (**Figure S7B**). In addition, we investigated whether PGA/Alum enhances cellular immune responses specific to $OVA_{257-264}$ peptides which are MHC class I-restricted peptide epitope of OVA. ELISPOT assay revealed that the number of $OVA_{257-264}$-specific IFN-γ-secreting cells was significantly higher in the PGA/Alum-OVA group than in the OVA or alum-OVA groups ($P < 0.01$) (**Figure 4C**). Moreover, flow cytometry showed that the frequency of $OVA_{257-264}$ tetramer-positive CD8$^+$ T cells was significantly increased in PGA/Alum-OVA compared to other groups ($P < 0.01$) (**Figure 4D**). These findings indicate that PGA/Alum enhances antigen-specific cellular immune responses, such as ADCC and CTL activities.

As immunological memory is a critical goal for effective vaccination, we examined the long-term immunity of mice treated with OVA, alum-OVA, γ-PGA-OVA, or PGA/Alum-OVA. As shown in **Figure 4E**, on day 180 after the final immunization, the PGA/Alum-OVA group showed a significantly higher IgG level than the other groups ($P < 0.01$). Notably, the increased IgG level of the PGA/Alum-OVA group was similar on days 14 and 180 after the final immunization, whereas those of the alum-OVA and γ-PGA-OVA groups had decreased to the baseline level by day 180 after the final immunization. The frequency of $OVA_{257-264}$-specific IFN-γ-secreting cells was also higher in the PGA/Alum-OVA group than in the groups treated with OVA or alum-OVA ($P < 0.01$) (**Figure 4F**). The PGA/Alum-OVA group also exhibited higher IgG1 and IgG2b levels (**Figure S8A**), greater percentages of both plasma cells and memory B cells (**Figure S8B**), more ADCC activity (**Figure S8C**), and a higher frequency of $OVA_{323-339}$ peptide-specific IL-4-secreting cells (**Figure S8D**) on day 180 after the final immunization. Taken together, these results demonstrate that PGA/Alum could be a potential adjuvant capable of enhancing humoral and cellular immunity and inducing persistent long-term immunity.

PGA/Alum Substantially Enhances the Protective Efficacy of Influenza Vaccine Antigen

To evaluate whether PGA/Alum improves the protective efficacy of a vaccine antigen, we investigated the effect of PGA/Alum on the immunogenicity and efficacy of pH1N1 split vaccine antigen. Mice were i.m. immunized with the pH1N1 split vaccine antigen (A/California/7/2009 NYMC X-179A H1N1) mixed with PGA/Alum (PGA/Alum-vaccine), alum (alum-vaccine), or γ-PGA (γ-PGA-vaccine) on days 0 and 14. The mice immunized with vaccine antigen alone or PBS were used as negative controls. Fourteen days after the final immunization, the immunized mice were intranasally (i.n.) challenged with a lethal dose (50 LD_{50}) of pH1N1 virus (A/California/04/09). As shown in **Figures 5A,B**, the mice of the PGA/Alum-vaccine group showed 100% survival without considerable body weight loss for 14 days after this viral challenge. In contrast, the mice immunized with alum-vaccine and γ-PGA-vaccine showed severe body weight loss and were only partially protected, exhibiting survival rates of 16.7 and 33.3%, respectively. The mice immunized with PBS or vaccine alone had 0% survival. As viral clearance from the infected lung is a crucial indicator of the protective efficacy of a vaccine, we determined viral titers in lung homogenates on days 3 and 7 post-infection. As expected, the PGA/Alum-vaccine group exhibited viral clearance on day 7 post-challenge, whereas the other groups continued to exhibit high viral titers at this point (**Figure 5C**). Our findings indicate that PGA/Alum substantially enhances the protection efficacy of the pH1N1 split vaccine antigen against pH1N1 virus challenge.

To confirm the adjuvant effect of PGA/Alum for the influenza vaccine, we examined IgG titers, hemagglutination-inhibition (HI) titers, ADCC, and CTL activities. As shown in **Figure 5D**, the titers of influenza antigen-specific IgG, IgG1, and IgG2b were higher in the sera obtained from the mice of the PGA/Alum-vaccine group than those obtained from the other groups. The HI titers were also substantially increased in the sera of the mice from the PGA/Alum-vaccine group (359 ± 180 geometric mean titer [GMT]) compared to those of the other groups (90 ± 45 GMT for alum-vaccine, 9 ± 6 GMT for γ-PGA-vaccine, 20 ± 14 GMT for vaccine, and 1 ± GMT for PBS) (**Figure 5E**), which indicates that PGA/Alum confers neutralizing humoral immunity. Next, when ADCC activity was analyzed by measuring lysis of pH1N1-infected MDCK cells through co-culture of NK cells and the sera Abs of immunized mice, the sera Abs of PGA/Alum-vaccine group had significantly higher ADCC

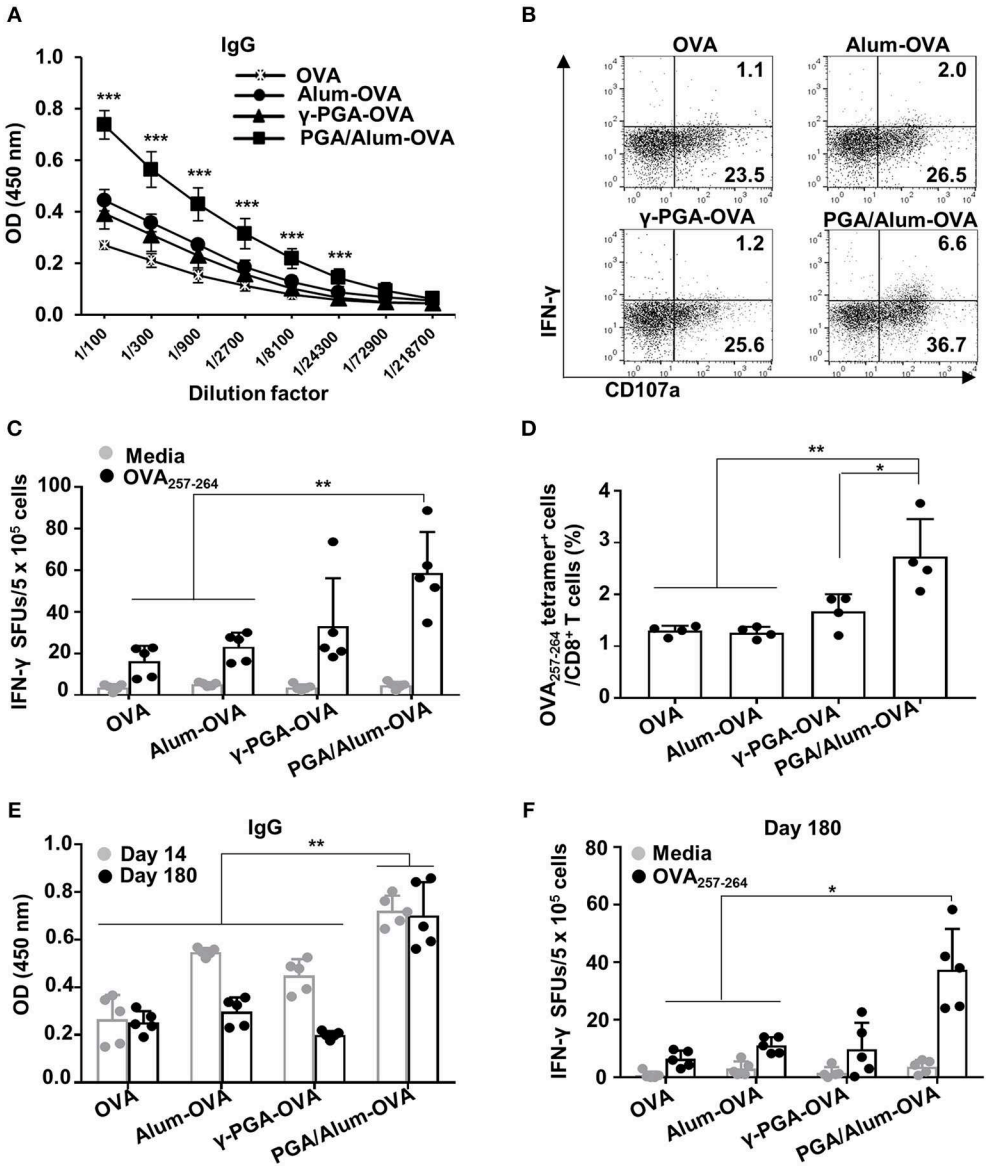

FIGURE 4 | PGA/Alum enhances OVA-specific humoral and cellular immune responses. C57BL/6 mice ($n = 5$ per group) were i.m. immunized with 10 μg OVA protein combined with 400 μg alum, 400 μg γ-PGA, or 800 μg PGA/Alum on days 0, 14, and 28. Fourteen **(A–D)** and 180 **(E,F)** days after the last immunization, sera and splenocytes were obtained from the immunized mice. **(A)** Serum levels of OVA-specific IgG were measured via ELISA. **(B)** OVA-coated plates were incubated with heat-inactivated serum samples (1 h at 56°C), washed, and then further incubated for 5 h with naïve NK cells in the presence of PE-conjugated anti-CD107a Ab, monensin, and brefeldin A. The cells were fixed, permeabilized, and stained with APC-conjugated anti-IFN-γ Ab. Activation of NK cells was assessed by flow cytometry. **(C)** Splenocytes were stimulated with $OVA_{257-264}$ peptide for 3 days, and the number of $OVA_{257-264}$-specific IFN-γ spot forming units (SFUs) was determined by an ELISPOT assay. **(D)** Frequency of $OVA_{257-264}$ tetramer$^+$ CD8$^+$ T cells was determined in the splenocytes using flow cytometry. **(E)** The level of OVA-specific IgG at days 14 and 180 post-immunization and **(F)** the number of $OVA_{257-264}$-specific IFN-γ SFUs on day 180 were determined by ELISA and an ELISPOT assay, respectively. Data are representative of three independent experiments with similar results. Statistically significant differences were identified via ANOVA/Bonferroni; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

activities (58.2 ± 0.5%) compared with those of the other groups (18.5 ± 0.2% for alum-vaccine, 32.8 ± 3.6% for γ-PGA-vaccine, 14.7 ± 3.5% for vaccine, and 8.2 ± 3.1% for PBS) (**Figure 5F**). Moreover, ELISPOT assay showed that the mice of the PGA/Alum-vaccine group yielded significantly more pH1N1 virus-specific IFN-γ-secreting cells than the other groups ($P < 0.05$) (**Figure 5G**). The percentages of IFN-γ-secreting CD4$^+$ and CD8$^+$ T cells were also higher in the PGA/Alum group than in the other groups (**Figure S9**). Taken together, our results suggest that PGA/Alum enhances the protective efficacy of the influenza vaccine antigen by modulating influenza antigen-specific humoral and cellular immunity.

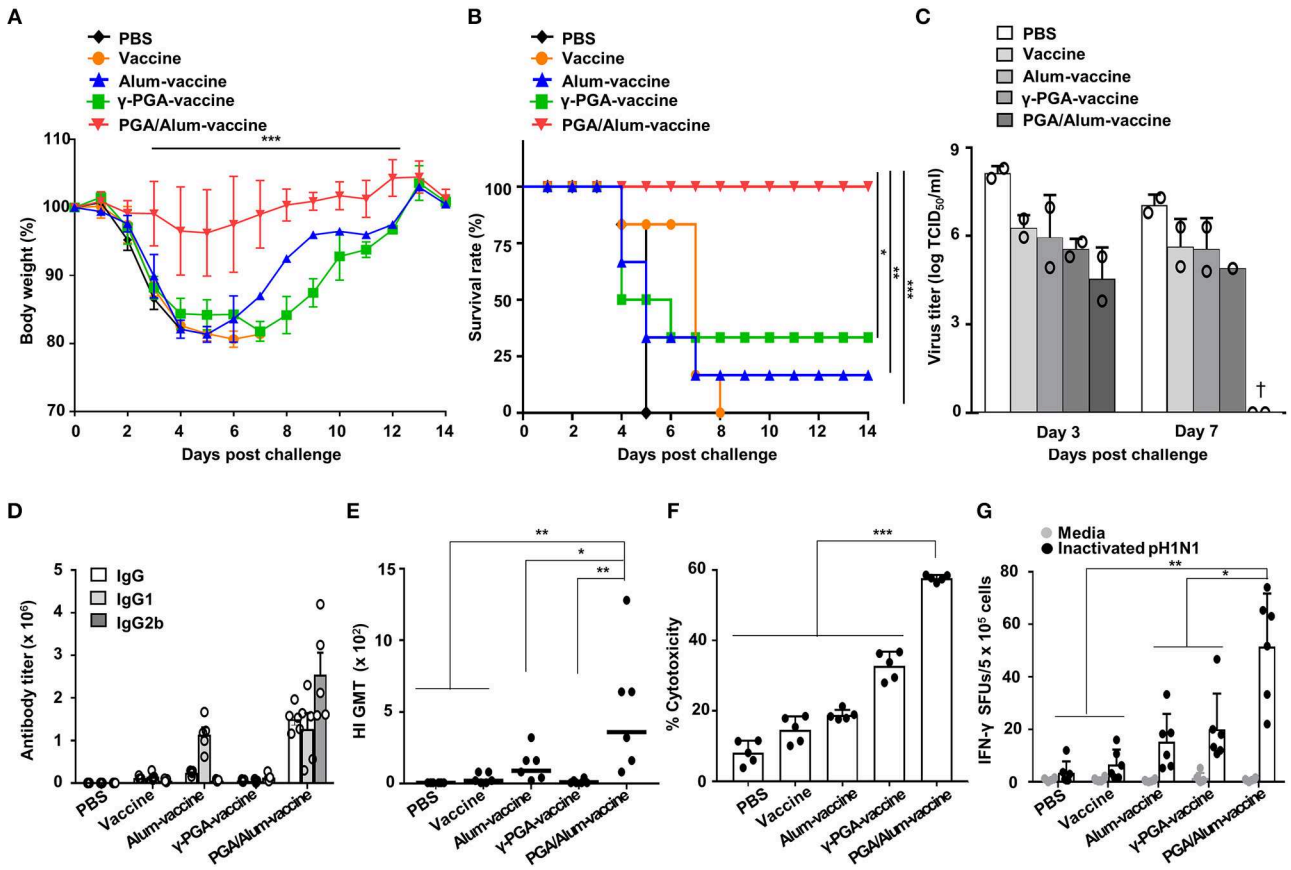

FIGURE 5 | PGA/Alum improves the protective efficacy of influenza vaccine antigen. C57BL/6 mice ($n = 6$ per group) were i.m. immunized with the 0.05 μg pH1N1 split vaccine antigen combined with 400 μg alum, 400 μg γ-PGA, or 800 μg PGA/Alum on days 0 and 14. Two weeks after the final administration, the mice were i.n. challenged with 50 LD_{50} pH1N1 virus. **(A)** Body weight changes and **(B)** survival rates were monitored for up to 14 days post-infection (dpi). Each data point represents an average percentage. Statistically significant differences were identified via the log-rank test. **(C)** Lung homogenates of each group were obtained at 3 and 7 dpi. Viral titers were determined using the pooled lung homogenates in duplicate and are expressed as $\log_{10}TCID_{50}$/ml (†, virus clearance). **(D)** Sera ($n = 5$ per group) were collected before the viral challenge, and endpoint titers of vaccine-specific Abs are expressed as the mean ± SD. **(E)** Serum HI titers against a pH1N1 virus were measured. The lines indicate geometric means, and negative titers were assigned a value of 5 for calculation. **(F)** The pH1N1-infected MDCK cells were incubated with naïve NK cells in the presence of the sera from immunized mice, and cytotoxicity was assessed by LDH assay. **(G)** The number of influenza virus antigen-specific IFN-γ-secreting splenocytes was determined via ELISPOT assays. Data are representative of at least three independent experiments. Statistically significant differences were identified by ANOVA/Bonferroni; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

PGA/Alum Substantially Improves the Cross-Reactive Immunity of Influenza Vaccine Antigen

Given our observations that PGA/Alum increased the ADCC and CTL activities responsible for cross-reactivity against heterologous influenza virus, we speculated that PGA/Alum might enhance the cross-protective efficacy of pH1N1 vaccine antigen. To evaluate the impact of PGA/Alum on this cross-reactivity, mice were immunized the pH1N1 split vaccine antigen mixed with alum, γ-PGA, or PGA/Alum and then challenged with A/Puerto Rico/8/34 (H1N1). Body weight decreased similarly across all groups until 6 days after the viral challenge, but was recovered faster by the PGA/Alum-vaccine group compared to the other groups (**Figure 6A**). Importantly, all mice of the PGA/Alum-vaccine group showed 100% survival, whereas only partial survival (40%) was observed in the alum-vaccine and γ-PGA-vaccine groups (**Figure 6B**). To ascertain the effect of PGA/Alum on cross-protective immunity, we challenged the vaccinated mice with heterosubtypic influenza A virus, H3N2. Mice of the PGA/Alum-vaccine group efficiently recovered the body weight lost following viral challenge and showed 80% survival. In contrast, only 20% of mice in the alum-vaccine or γ-PGA-vaccine groups survived, and no survival was seen in the PBS or vaccine-alone groups (**Figures 6C,D**).

To better understand the mechanisms underlying PGA/Alum-enhanced cross-reactive protection, we examined cross-reactive ADCC and CTL activities. Our ADCC assay revealed that the cytolysis of heterologous influenza virus (H1N1 or H3N2)-infected MDCK cells was significantly increased by co-culture with naïve NK cells and serum Abs obtained from the PGA/Alum-vaccine group (25.6 ± 1.9% for H1N1-infected MDCK cells and 31.1 ± 1.3% for H3N2-infected

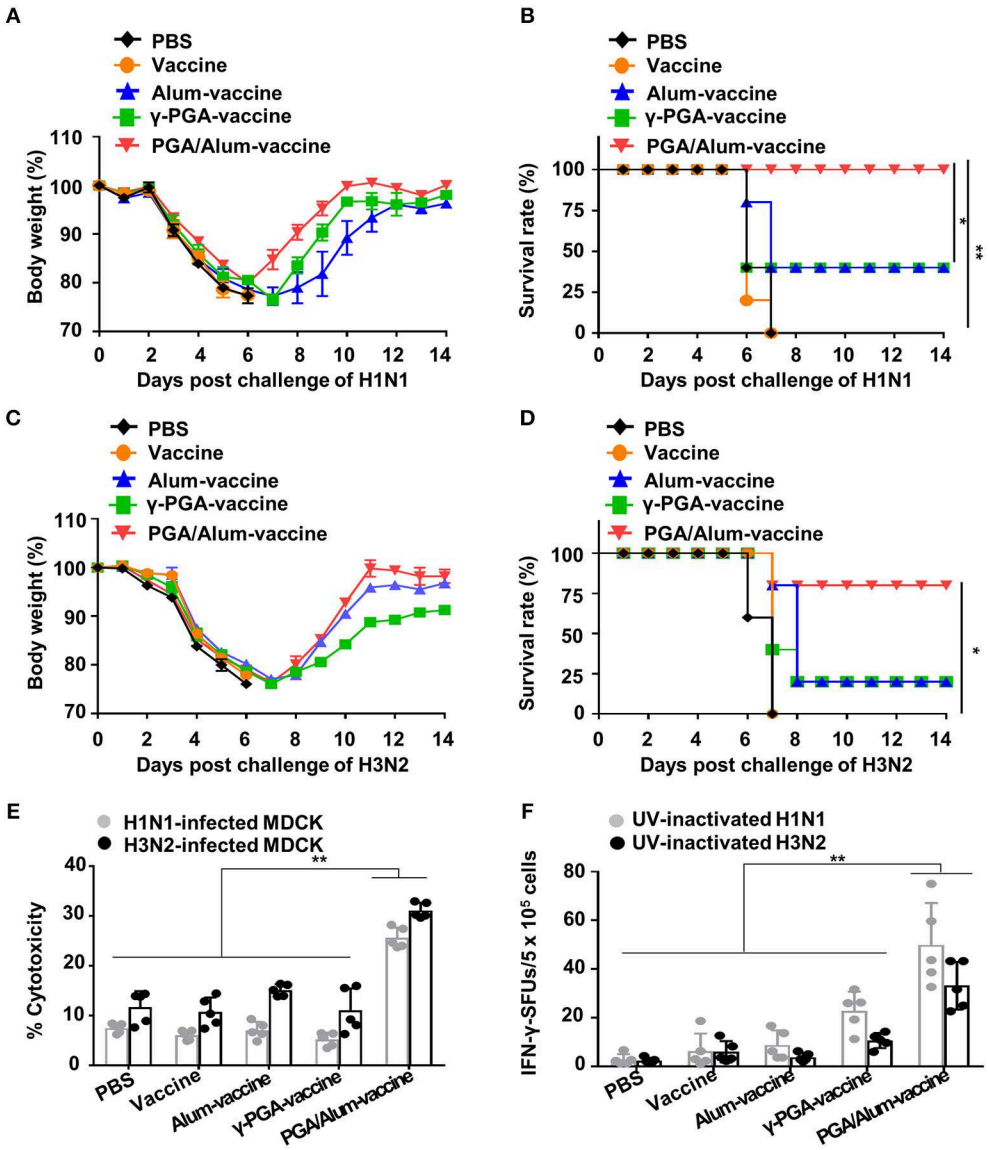

FIGURE 6 | PGA/Alum enhances cross-protective efficacy of pH1N1 vaccine antigen. C57BL/6 mice ($n = 5$ per group) were vaccinated i.m. with 0.5 μg the pH1N1 split vaccine antigen together with 400 μg alum, 400 μg γ-PGA, or 800 μg PGA/Alum on days 0 and 14. Two week after the last immunization, the mice were i.n. challenged with 10 LD_{50} H1N1 virus (A/Puerto Rico/8/1934) **(A,B)** or 10 LD_{50} H3N2 viruses **(C,D)**. Body weight and survival rates were monitored for 14 days. **(E,F)** Before viral challenge, sera and splenocytes were harvested from the immunized mice. **(E)** ADCC activity was determined by measuring lysis of H1N1- or H3N2-infected MDCK cells by co-culture of sera from the vaccinated mice and naïve NK cells. **(F)** Splenocytes were stimulated with UV-inactivated H1N1 or UV-inactivated H3N2 for 3 days, and the number of IFN-γ^+ SFUs was determined by an ELISPOT assay. Statistically significant differences were identified by one-way ANOVA/Bonferroni or log-rank test (for survival); $^*P < 0.05$ and $^{**}P < 0.01$.

MDCK cells), but not serum Abs obtained from mice exposed to alum-vaccine (6.9 ± 1.3% and 15.1 ± 1%, respectively), γ-PGA-vaccine (5.2 ± 1.2 and 11.1 ± 3.5%, respectively), vaccine alone (6.0 ± 0.8 and 10.7 ± 2.9%, respectively), or PBS (7.5 ± 0.9 and 11.8 ± 2.9%, respectively) (**Figure 6E**). To clearly elucidate Ab-mediated contribution of PGA/Alum-enhanced heterosubtypic cross-protection, we further performed *in vivo* protection assay as previously described (34, 35). Heat-inactivated sera from the immunized mice were mixed with H3N2 virus, and then the mixture was i.n. challenged to Balb/c mice. As shown in **Figure S10**, at day 14 post-infection, mice exposed to the mixture of H3N2 virus and PGA/Alum-vaccine-immunized sera had 20% survival rate, whereas all mice of other groups died (0% survival rate), indicating that PGA/Alum-enhanced Ab production may partially contribute to heterosubtypic cross-protection. As the ADCC-mediating Abs are thought to recognize the highly conserved stalk domain of hemagglutinin (HA) on the influenza virus, thereby leading

to cross-protection (36), we tested ADCC-mediated NK cell activation by incubating the serum Abs with the HA stalk protein. Our results revealed that HA stalk protein-preincubated serum Abs from the PGA/Alum-vaccine group significantly increased the percentages of IFN-γ^+ NK and CD107$^+$IFN-γ^+ NK cells (**Figure S11**), indicating that the PGA/Alum-induced Abs effectively bind to the HA stalk domain and subsequently activate NK cells. Additionally, we performed ELISAs to measure antibodies against the HA stalk using a recombinant HA stalk protein from A/Puerto Rico/8/1934 (H1N1) which share 94% identity to A/California/04/09 (pH1N1) and 50% identity to A/Hong Kong/1/68 (H3N2) virus. HA stalk-specific IgG level was significantly higher in the sera from the PGA/Alum-vaccine group than those in other groups, although the pH1N1 vaccine alone group very little elicited H1 HA stalk-specific IgG level (**Figure S12A**). We further tested H3 ELISA using a recombinant HA1 protein of H3N2 virus to investigate whether cross-reactive neutralizing Abs contribute to cross-protection against H3N2 virus. None of the pH1N1 vaccine antigen-immunized groups elicited H3 HA1-specific IgG titers (**Figure S12B**). Moreover, the sera Abs from all groups had no cross HI reactivity against heterosubtypic H3 virus (**Figure S12C**). These results suggest that PGA/Alum elicits HA stalk Abs but not cross-reactive neutralizing Abs. Furthermore, an ELISPOT assay revealed that PGA/Alum increased cross-reactive CTL activity. As shown in **Figure 6F**, the number of H1N1-specific IFN-γ-secreting splenocytes was at least 2-fold higher in the PGA/Alum-vaccine group (50 ± 17 SFUs) compared to the other groups (23 ± 8 SFUs for γ-PGA-vaccine, 9 ± 6 SFUs for alum-vaccine, 6 ± 7 SFUs for vaccine alone, and 3 ± 2 SFUs for PBS). Moreover, the number of H3N2-specific IFN-γ-secreting cells was at least 3-fold higher in the PGA/Alum-vaccine group (33 ± 10 SFUs) than in the other groups (11 ± 3 SFUs for γ-PGA-vaccine, 4 ± 2 SFUs for alum-vaccine, 6 ± 4 SFUs for vaccine alone, and 2 ± 1 SFUs for PBS) ($P < 0.01$). Collectively, these findings demonstrate that PGA/Alum could enhance cross-protection by improving cross-reactive ADCC and CTL activities.

DISCUSSION

Currently, researchers are seeking to develop new adjuvants that increase vaccine-induced protection against infectious diseases. The use of TLR agonists as vaccine adjuvants is considered a promising means to improve vaccine efficacy, because TLR4 activates innate immune responses and subsequently augments adaptive immune responses by enhancing Th1-biased responses (14, 15). The combination of a TLR4 agonist with a Th2 adjuvant (e.g., alum) could be a beneficial strategy for tailoring immune responses through synergistic effects. The value of this strategy was emphasized by the recent approval for human use of AS04, which comprises MPL (a TLR4 agonist) absorbed on alum (37, 38). However, the purification of MPL requires extensive chemical modification of biologically derived LPS. There is a large batch-to-batch variability, the cost is prohibitive, and there are safety concerns. To address these limitations, we set out to replace MPL with the safe and cost-effective biomaterial, γ-PGA, to generate the new combination of PGA/Alum and demonstrated its efficacy as an adjuvant, as summarized in **Figure 7**. We propose that the synergistic effect of PGA/Alum is mediated mainly by γ-PGA-induced innate immune activity plus alum-induced depot effect. γ-PGA activates innate immune responses including increases of costimulatory molecule expression and cytokine production of APCs. Concomitantly, alum induces vaccine antigen depots capable of enhancing antigen presentation by activating APCs. Also, antigen loading capacity of PGA/Alum may be enhanced by biopolymeric property of γ-PGA and antigen absorption capacity of alum. The combined effect of γ-PGA and alum could robustly provoke Th1 immune responses to enhance CTL activity and highly increase humoral immune responses, thereby leading to improved vaccine efficacy.

An ideal vaccine adjuvant should have a broad-spectrum of safety. We observed that PGA/Alum had very little cytotoxic effect on cells *in vitro*, although alum itself induced cytotoxicity. We speculate that the combination of γ-PGA with alum may be able to block the cytotoxicity of alum. It has been reported that immunization of MF59-adjuvanted influenza vaccine induced adverse reaction (e.g., redness and swelling at the injection site) compared to unadjuvanted vaccine in children (39, 40). By visual observation, administration of PGA/Alum induced no redness and swelling at the injection site (data not shown). Especially, no significant changes of body temperature as well as inflammatory cytokines were observed in the sera of the vaccinated mice. These findings suggest that PGA/Alum may be safe to use. In addition, γ-PGA can be produced on an industrial scale without complex requirements, and thus does not have the limitations associated with the complicated process required to manufacture MPL. A side-by-side comparison of adjuvants including MF59 and AS04 is important to elucidate the adjuvant effect of PGA/Alum. Unfortunately, we cannot compare the adjuvant effect of AS04 and PGA/Alum, because AS04 is unavailable for research use. In the case of MF59, we made squalene-based oil-in-water nano-emulsion with a formulation similar to MF59 (MF59-like adjuvant). In influenza vaccine experiments, PC nanogel [γ-PGA/chitosan nanogel adjuvant published in (18)] had similar protective efficacy with MF59-like adjuvant but lower than that of PGA/Alum (unpublished data). We also observed that MF59-like adjuvant did not induce cell-mediated immunity against the influenza vaccine antigen by IFN-γ ELISPOT assay but PGA/Alum robustly enhanced cell-mediated immunity (unpublished data). Therefore, we imply that PGA/Alum could act as a more potent adjuvant than MF59.

Activated DCs critically contribute to antigen processing and presentation. The activation of DCs and their delivery of antigen to LNs are crucial for the ability of a vaccine to effectively initiate innate and adaptive immune responses (41). Our results showed that PGA/Alum induces DC activation through TLR4 signaling, as assessed by increases in the levels of costimulatory molecules and pro-inflammatory cytokines, as well as enhancement of antigen processing. PGA/Alum robustly enhanced both antigen trafficking and the migration of antigen-loaded DCs from the injection sites to dLNs, which is

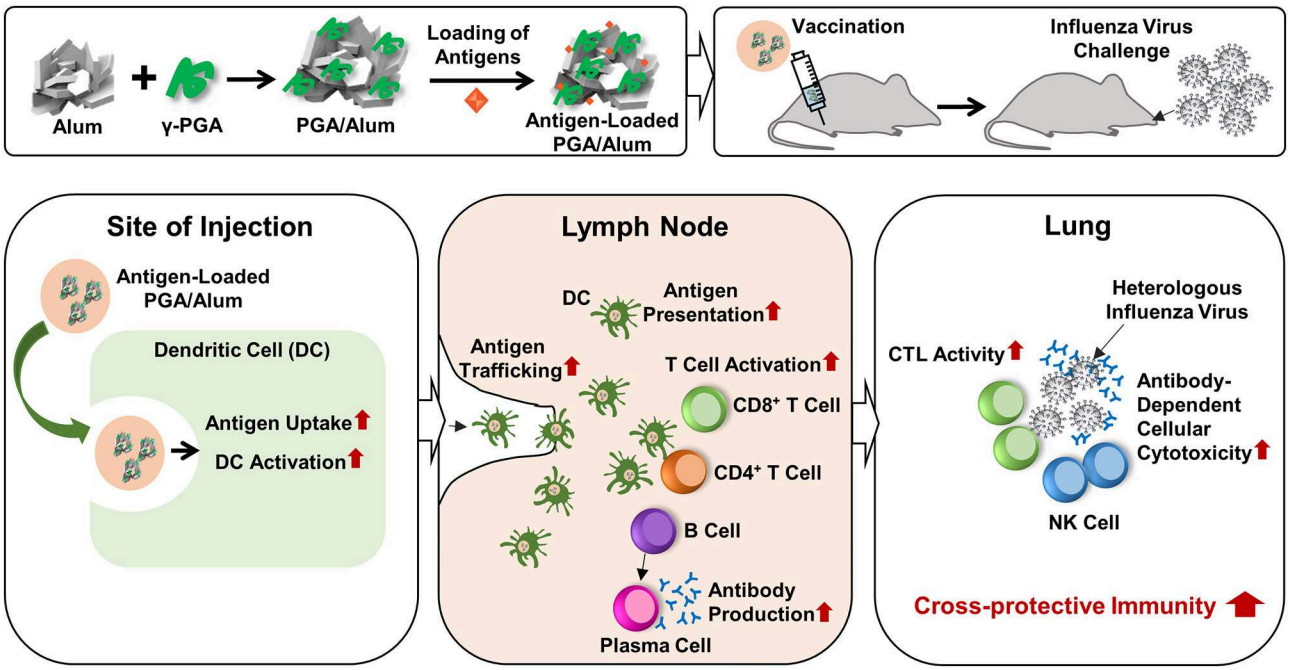

FIGURE 7 | Schematic illustration of the fabrication of PGA/Alum and its action mechanism. PGA/Alum induces the innate immunity such as antigen uptake and delivery by DCs to lymph nodes, thereby leading to the enhanced antigen-specific cellular and humoral immunities. Consequently, PGA/Alum improves the cross-reactive immunity of influenza vaccine antigens against heterologous influenza virus, with increases of CTL and ADCC activities.

consistent with previous findings obtained with the MPL-based adjuvants, AS04 and AS01 (37, 38). Our immunofluorescent microscopic analysis further revealed that PGA/Alum increased antigen accumulation in the subcapsular, cortical, and medullary sinus regions of dLNs, indicating that antigen presentation in LNs could be facilitated by PGA/Alum. Circulating immature DCs reach inflamed tissues by following the chemoattractant gradient to uptake antigens and then migrate to dLNs to initiate adaptive immunity (29). Thus, it is relevant that PGA/Alum was found to robustly enhance not only the recruitment of DCs to injection sites and dLNs but also the migration of antigen-loaded DCs to dLNs. Consistent with previous studies in which mice were injected with other adjuvants, including AS04, AS01, and AS03 (37, 38, 42), PGA/Alum increased the levels of chemokines (MIP-1α, MIP-1β, and MCP-1) at injection sites and dLNs. We also found that the expression of CCR7 on DCs, which is responsible for their migration to LNs, was increased by PGA/Alum. Notably, this has not been reported for AS04. Thus, the ability of PGA/Alum to enhance DC migration by increasing chemokine levels and CCR7 expression might provide insight into the action mechanisms of this vaccine adjuvant. Together, our findings demonstrate that PGA/Alum can act as a potent adjuvant capable of activating innate immune responses, including DC activation, DC migration, and antigen trafficking.

Our proof-of-concept experiments demonstrated that PGA/Alum efficiently enhances the humoral and cellular immune responses specific to both OVA (a model antigen) and the influenza vaccine antigen (a vaccine antigen of a representative infectious disease). Importantly, we found that PGA/Alum significantly increased ADCC, which has recently been shown to induce effective protection against various viruses, including Ebola (9), human immunodeficiency virus (43, 44), Epstein-Barr virus (45), and influenza viruses (8, 32). Our results from an ADCC assay performed using mouse serum revealed that PGA/Alum enhanced ADCC activity. As ADCC is known to be initiated by the IgG2 subclass in mice (32), it is notable that we observed a significant induction of IgG2b in the sera of mice immunized with the antigen mixed with PGA/Alum. Because γ-PGA induces Th1 responses (17, 46–48), which are associated with the induction of IgG2b (49), our results suggest that γ-PGA acts synergistically with alum in PGA/Alum to elevate the production of IgG2b and thereby enhance ADCC.

Influenza A viruses exist as several subtypes, and new viruses can emerge due to point mutations (e.g., antigenic drift) or genetic reassortments between different viral subtypes (e.g., antigen shift), potentially leading to influenza epidemics and pandemics (50). The cross-reactivity of an influenza vaccine is essential for its ability to broadly protect against antigenically drifted influenza viruses. Our present results reveal that PGA/Alum enhanced the protective efficacy of the influenza pH1N1 vaccine against homologous virus. Importantly, PGA/Alum-adjuvanted pH1N1 vaccine exhibited improved cross-protection against both heterologous influenza virus (e.g., H1N1) and heterosubtypic virus (e.g., H3N2). In contrast,

cross-protection was not observed in mice immunized with alum- or γ-PGA-adjuvanted pH1N1 vaccine. As cross-reactive immunity is primarily mediated by CTLs, which recognize broadly conserved epitopes shared by influenza A virus subtypes (50), our results indicate that PGA/Alum-adjuvanted pH1N1 vaccine drastically enhanced cross-reactive CTL activities against H1N1 and H3N2 viruses. In this experiment, we used a reassortant H3N2 virus carrying HA and NA genes from the A/Hong Kong/1/68 and internal genes from A/Puerto Rico/8/34. Cell mediated immunity against conservation of internal proteins may contribute to PGA/Alum-enhanced heterosubtypic protection. In addition, PGA/Alum enhanced ADCC, which is considered to be the crucial function of non-neutralizing Abs in cross-reactive immunity, and pH1N1 vaccine-PGA/Alum-induced sera Abs had cross-reactive ADCC activity against H1N1 and H3N2 viruses. Consistent with previous reports showing that ADCC-mediating Abs bind to the conserved HA stalk domain of the influenza virus (10, 51), PGA/Alum increased the level of the HA stalk Abs but not cross-reactive neutralizing Abs of heterologous virus (**Figure S12**). *In vivo* cross-protection assay conferred that PGA/Alum-enhanced heterosubtypic cross-protection may be mainly cell-mediated and partially by humoral immunity, similar with a previous report (**Figure S10**) (52). Thus, PGA/Alum might help improve the protective efficacy and cross-protection of the influenza vaccine, and its use as an adjuvant could help resolve the limitations of the current influenza vaccines. Given that ADCC and CTL activities have been suggested to contribute to protecting elderly individuals against influenza virus infection (53, 54), our results suggest that PGA/Alum could be used as a vaccine adjuvant for older people with dysregulated immune responses. Taken together, our findings indicate that PGA/Alum may be a promising candidate as a vaccine adjuvant for preventing diseases caused by influenza viruses and other infectious agents.

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

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the KRIBB and were performed according to the Guidelines for Animal Experiments of the KRIBB.

AUTHOR CONTRIBUTIONS

HP designed and supervised the experiments, analyzed results, and wrote the manuscript. QN, CK, JK, WL, and JY performed the experiments. QN, CK, and JY analyzed the experiments and drafted the manuscript. MS contributed to produce γ-PGA and discuss the results. WL and JJ contributed to analyze chemical characterization of the PGA/Alum.

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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.2019.01604/full#supplementary-material

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Hepatitis E Virus (HEV)-Specific T Cell Receptor Cross-Recognition: Implications for Immunotherapy

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T cell immunotherapy is a concept developed for the treatment of cancer and infectious diseases, based on cytotoxic T lymphocytes to target tumor- or pathogen-specific antigens. Antigen-specificity of the T cell receptors (TCRs) is an important selection criterion in the developmental design of immunotherapy. However, off-target specificity is a possible autoimmunity concern if the engineered antigen-specific T cells are cross-reacting to self-peptides *in-vivo*. In our recent work, we identified several hepatitis E virus (HEV)-specific TCRs as potential candidates to be developed into T cell therapy to treat chronic hepatitis E. One of the identified TCRs, targeting a HLA-A2-restricted epitope at the RNA-dependent RNA polymerase (HEV-1527: LLWNTVWNM), possessed a unique multiple glycine motif in the TCR-β CDR3, which might be a factor inducing cross-reactivity. The aim of our study was to explore if this TCR could cross-recognize self-peptides to underlay autoimmunity. Indeed, we found that this HEV-1527-specific TCR could also cross-recognize an apoptosis-related epitope, Nonmuscle Myosin Heavy Chain 9 (MYH9-478: QLFNHTMFI). While this TCR had dual specificities to both viral epitope and a self-antigen by double Dextramer binding, it was selectively functional against HEV-1527 but not activated against MYH9-478. The consecutive glycine motif in β chain may be the reason promoting TCR binding promiscuity to recognize a secondary target, thereby facilitating cross-recognition. In conclusion, candidate TCRs for immunotherapy development should be screened for autoimmune potential, especially when the TCRs exhibit unique sequence pattern.

Keywords: CD8+ T cells, cross-reactivity, T cell therapy, immunotherapy, T cell receptor (TCR), TCR redirection, hepatitis E virus (HEV)

INTRODUCTION

T cell immunotherapy was initially developed as a cancer treatment in late stage melanoma, to target tumor-associated antigens with the aim to control or eliminate tumor growth (1). This approach is formulated based on immune-mediated T cell responses, more specifically, the involvement of cytotoxic T lymphocytes harboring T cell receptors (TCR) that have specificities to target tumor antigens. Lately, the principle of T cell immunotherapy has been applied to treat infectious diseases, keeping the same fundamental concept to target pathogenic antigens by adoptive transfer of effector CD8+ T cells expressing antigen-specific TCRs. The immune responses triggered by adoptive transfer of antigen-specific T cells are proven effective in clinical applications, as reconstituted cellular immunity prevented human cytomegalovirus (CMV) (2), Epstein–Barr virus (EBV) (3), and adenovirus (4) infections in patients who underwent allogenic hematopoietic stem cell transplant. Recently, hepatitis B virus (5, 6) and human papilloma virus (7, 8) were also investigated for virus-associated malignancies to which cure is not available.

We intended to explore the aptitude of T cell therapy in treating chronic hepatitis E, using hepatitis E virus (HEV)-specific CD8+ T cells, since robust and diverse CD8+ T cell responses are crucial in viral control (9). We aimed to address an unmet need in chronic hepatitis E, as there is currently no approved therapy (10), and off-label treatments are associated with severe side effects. We proposed that immunotherapy based on engineered T cells targeting HEV could be a novel approach to treat persistent HEV infection in solid organ transplant patients who are immunosuppressed.

Our previous work had identified promising HEV-specific TCR candidates in healthy donors (who may have recovered from previous HEV infections) and patients with acute hepatitis E for T cell-based therapy (11). Interestingly, one of the HEV-specific T cell population isolated from a healthy donor had a TCR repertoire comprised of two α chains and one β chain containing multiple glycines.

This unique TCR repertoires prompted us to scrutinize its potential cross-reactive responses because oligoclonal TCR with dual alpha was first described by Padovan et al. in the early 1990 (12), whereby each of the α chain could pair up with the single β to form two independent TCRs (e.g., α1β and α2β) on the same T cell, each with their respective target peptides. Hence, dual alpha (accordingly, dual specificities) could contribute to autoimmune phenotype (13, 14). In addition, it has been suggested that multiple glycines motif in the TCR may induce its binding promiscuity to another epitope, thus facilitating cross-reactivity (15).

In the clinical setting of immunotherapy using T cells expressing target-specific TCR, cross-reactivity of TCR could be an autoimmune concern due to probable off-target specificity *in-vivo*. One example is the cross-reactive MAGE A3 tumor antigen-specific TCR, recognizing a second target (a cardiac peptide), triggering cardiac arrest in two clinical trial patients, both of whom died within 1 week of receiving the infusion of TCR-transduced T cells (16). Further investigation led to direct evidence of such cross-reactivity, which was not possible to be anticipated using pre-clinical models (e.g., cell lines and mice model), due to the unique expression profile of this cardiac peptide in mature human heart (17).

On the contrary, our proposal of HEV-specific TCR is targeting a non-self/viral peptide. Nevertheless, we implored to investigate its autoreactive potential before advancing it for further development, as a precaution.

Screening the entire ligandome of self-peptides that this TCR might recognize is a daunting task. Therefore, we focused on specific groups of self-antigens that are related to viral infection. Rawson et al. discussed how effector T cells destined to undergo programmed cell death (apoptosis) are cleaved by proteolytic enzymes called caspases (18) could induce autoimmunity when some caspase-cleaved apoptotic products are cross-presented, thereby priming auto-reactive T cells (19). Such model was used to explain the disease pathogenesis of rheumatoid arthritis (20) and multiple sclerosis (21). A second probable source of self-antigens is derived from our hypothesis that HEV-specific T cells would reside in the liver to target infected hepatocytes. If these T cells were autoreactive, the condition would manifest in the form of autoimmune hepatitis (AIH). In fact, we have documented a higher HEV seroprevalence in AIH patients (22). Thus, epitopes of liver enzyme cytochrome P4502D6 (CYP2D6) that are found to be correlated to AIH disease progression were also included in our screening panel (23).

In this project, we aimed to study the possible cross-reactivity of a HEV-specific TCR repertoire that was proposed as a candidate in T cell therapy in order to address the clinical concern of off-target specificity affecting self-peptides.

RESULTS

HEV-Specific T Cell Receptor (TCR) Repertoire With Unique α and β Configuration

We recently identified in a healthy donor the HEV-specific CD8+ T cells targeting RNA-dependent RNA polymerase in Open Reading Frame 1 (RdRp in ORF1) of HEV genome, $HEV_{1527-1535}$ (denoted as HEV-1527 henceforth) with prominent T cell responses upon peptide stimulation (11). **Figure 1A** summarizes the discovery workflow of HEV-1527-specific CD8+ T cells, which we proposed as a candidate for T cell therapy. Briefly, CD8+ T cells isolated from a cohort of nine healthy donors (D1 to D9) were expanded in the presence of HEV overlapping peptide pools to screen for HEV-specific CD8+ T cell epitopes. Donor D2 showed strong immune responses against RdRp and the epitope was consequently narrowed down to HEV-1527. Dextramer bearing this epitope was synthesized to sort the HEV-1527-specific CD8+ T cells from D2, for T cell receptor (TCR) sequencing.

Next Generation Sequencing on TCR repertoires proved the presence of oligoclonal α chains (almost equal split between TCRAV12-02 and TCRAV04-01) and one dominant β chain (TCRBV04-02) containing

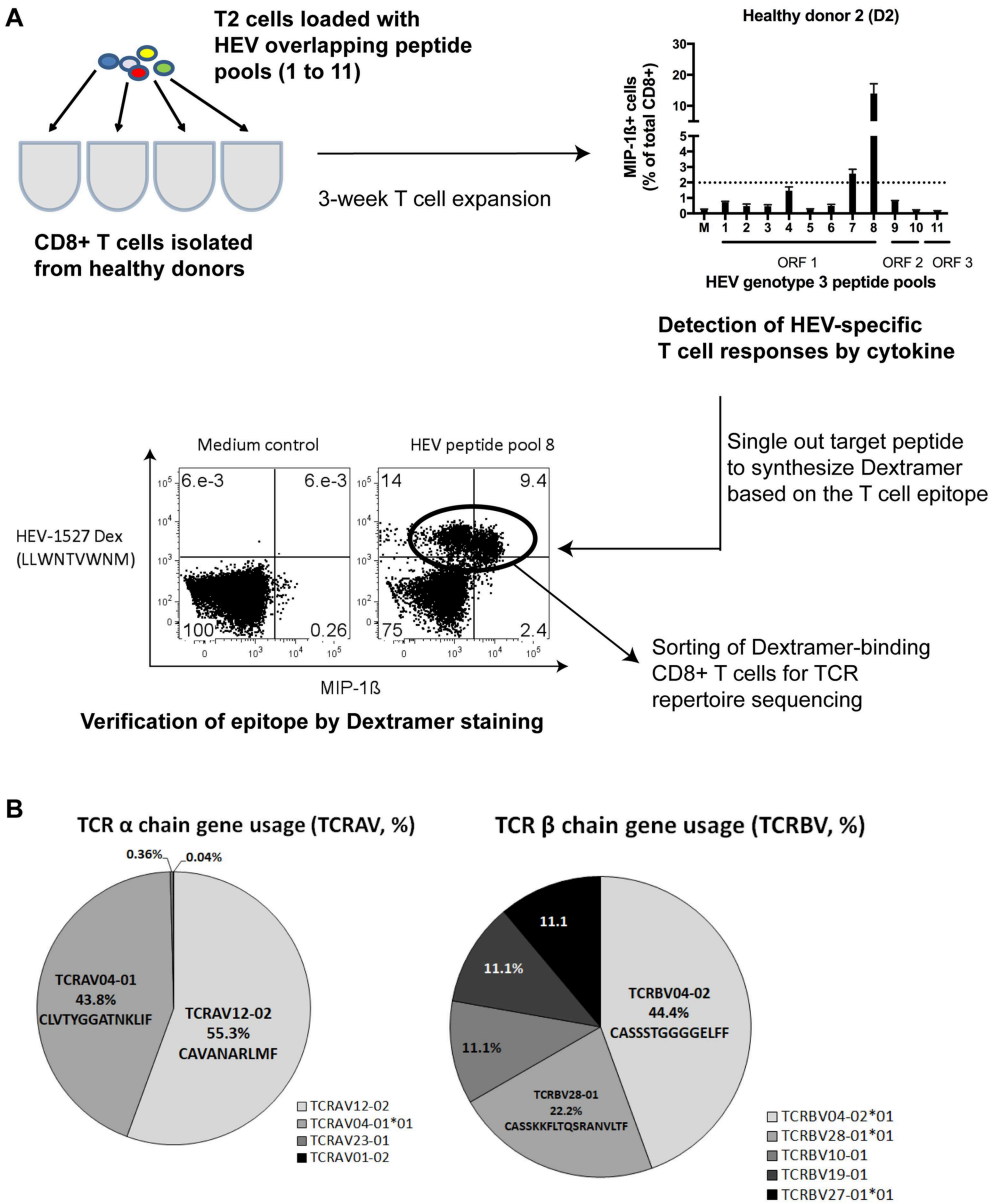

FIGURE 1 | HEV-specific T cell receptor (TCR) repertoires. **(A)** Simplified workflow illustrated how HEV-1527 epitope was identified from a healthy donor, D2, by HEV overlapping peptide pool screening and cytokine readout. **(B)** Next Generation Sequencing results of the TCR targeting HEV-1527, an epitope located at RNA-dependent RNA polymerase.

a block of consecutive glycine in the CDR3 region (**Figure 1B**).

Additionally, we repeated the sequencing using two different methods (Sanger and Deep sequencing) and proved that the HEV-1527-specific TCR was indeed oligoclonal in the TCR α chains. Both methods concluded the prevalence of the TCRBV04-02 clonotype, with consecutive 4–6 glycines in the CDR3 region (**Supplementary Figure 2**). Due to this unique combinatory of two TCR α chains and the presence of multiple glycines in CDR3, we decided to screen this T cell clone for possible cross-reactivity to self-antigens.

Screening of Apoptosis- and Cytochrome-Associated Self-Antigens

There are countless possibilities of self-antigen that this TCR might recognize, therefore we concentrated on screening epitopes with pathogenesis relevance in two main aspects: apoptosis-related epitopes that could be cross-presented to T cells inadvertently (18) and cytochrome (liver enzyme)-specific epitopes that are correlated with disease progression of autoimmune hepatitis (23). Five epitopes of each group were used for HLA-A*02:01 MHC Class I Dextramer synthesis

(**Supplementary Table 1**), either as nonamers or decamers, in line with the optimal peptide length for HLA-A*02:01 allele (24).

CD8+ T cells from donor D2 were expanded in the presence of HEV-1527 peptide again, in order to detect proliferation of cross-reactive T cells with specificity to either apoptosis or cytochrome epitopes by Dextramer staining. In addition to D2, from whom we discovered this unique αβTCR, we also included eight other healthy donors as control in the screening, to compare their responses to D2. As shown in **Figure 2A**, there was strong apoptotic-specific Dextramer staining in HEV-1527-expanded T cells from D2, but not from other donors. On the other hand, Dextramer staining of cytochrome-specific epitopes across all the donors were negligible.

The positive apoptotic epitope was subsequently singled out from the group of five by staining the Dextramers individually (**Figure 2B**). This epitope was derived from Nonmuscle Myosin Heavy Chain 9, $MYH9_{478-486}$, with the sequence of QLFNHTMFI (referred to as MYH9-478 hereafter). The sequence homology between HEV-1527 and MYH9-478 was shown in **Figure 2C**, with the matching two out of nine amino acids highlighted in red.

To further validate that these T cells could recognize two rather dissimilar peptides, we stained the HEV-1527-expanded T cells with two Dextramers simultaneously, one bearing HEV-1527 epitope and the other MYH9-478, and the double Dextramer staining promptly demonstrated that the T cells indeed had dual specificities, as evident by the double positive population (**Figure 2D**).

Cross-Reactivity Is Non-reciprocal

With the dual specificities proven in the previous experiment, we wanted to explore if the T cell cross-reactivity is reciprocal. To do this, HEV-1527 and MYH9-478 T cell lines were expanded separately and stained with HEV-1527 as well as MYH9-478 Dextramers as proliferation readout. As expected, HEV-1527 T cell line could bind both HEV-1527 and MYH9-478 Dextramers, yet MYH9-478 T cell line harbored specificity to neither epitope (**Figure 3A**).

Apart from T cell proliferation, we also characterized T cell function on the T cell lines generated from **Figure 3A**, by stimulating them with respective peptides during intracellular cytokine staining. As seen in **Figure 3B**, HEV-1527-expanded T cell line was functionally activated when stimulated by HEV cognate stimulated by HEV cognate peptide, but not by the apoptotic epitope. MYH9-478 T cell line did not respond to stimulation from either peptide (**Figure 3B**).

Furthermore, a range of peptide concentrations were tested in HEV-1527 T cell line to determine its sensitivity to peptide stimulation. Both cytokine production and a gradual reduction of Dextramer-binding T cell population were observed in HEV-1527 T cell line when stimulated by HEV-1527 (**Figure 3C**), whereas the T cells remained non-responsive to MYH9-478 peptide in this assay, regardless of the peptide concentration.

Higher Avidity of Cross-Reactive T Cells Toward the HEV Peptide

Thus far, our evidences showed that this T cell line had dual specificity to recognize two target peptides yet only functionally respond to one epitope. Hence, a sensitivity test was performed using various dilutions of Dextramer, to examine the T cell avidity. Starting from the recommended Dextramer quantity (denoted as 1x), various dilutions were prepared by adding diluent to the Dextramer to yield the indicated dilution factors. In 2x concentration, double the amount of Dextramer was used in staining the same number of cells. As depicted in **Figure 4A**, when sufficient Dextramer molecules were present, such as 1x and 2x, the binding of TCR to Dextramers plateaued at these saturating concentrations.

In contrast, at lower Dextramer dilutions (between 1/5 and 1/20), the TCR had higher avidity toward HEV-1527 as the Dextramer staining of HEV-1527 was higher than that of MYH9-478. Furthermore, the percentage of MYH9-478 and HEV-1527 Dextramer-binding T cells was presented in a ratio (MYH9-478/HEV-1527), which declined as the Dextramer concentration was decreasing which indicates a higher avidity of the TCR toward HEV-1527.

Such preference toward HEV-1527 rather than MYH9-478, may explain the lack of T cell response when stimulated by MYH9-478 peptide, as observed in **Figures 3B,C**. In fact, when T cells were expanded in the presence of both HEV-1527 and MYH9-478 peptides, the T cells selectively activated against HEV-1527 only (**Figure 4B**).

Functional Characterization of TCR Clonotypes Using TCR Redirection

Next, we investigated which of the TCR α (or both) was responsible for this cross-recognition and selective functionality phenomenon. To serve this purpose, TCR redirection assay was used. This is a mRNA-based method to generate engineered T cells bearing TCR of interest for *in-vitro* assays (25). Based on the TCR repertoire sequencing data, mRNA encoding the TCRs was synthesized and transfected into recipient effector T cells by electroporation. Gene optimization of TCR constant regions is done to prevent mispairing between the introduced and endogenous TCRs (26), and the TCR-redirected cells were used for analysis on day 1 post-transfection, as described (27).

Since the sequencing data indicated that there were mainly 4 or 5 glycines in the TCRBV04-02 clonotype, we wanted to ascertain if the number of glycine in the β CDR3 would affect TCR function. Hence, our TCR construct designs paired up each of the two α clonotypes with the β clone, as outlined in **Supplementary Figure 3**. Construct A consisted of TCRAV04-01 and construct B consisted of TCRAV12-02, each paired up with a β clone of 4 glycines. Construct C consisted of TCRAV04-01 and construct D consisted of TCRAV12-02, each paired up with a β clone of 5 glycines.

Post-redirection, the TCR-engineered T cells were stained with Dextramers, not only to evaluate the transfection efficiency, but also to assess the specificity of the TCRs. **Figure 5A**

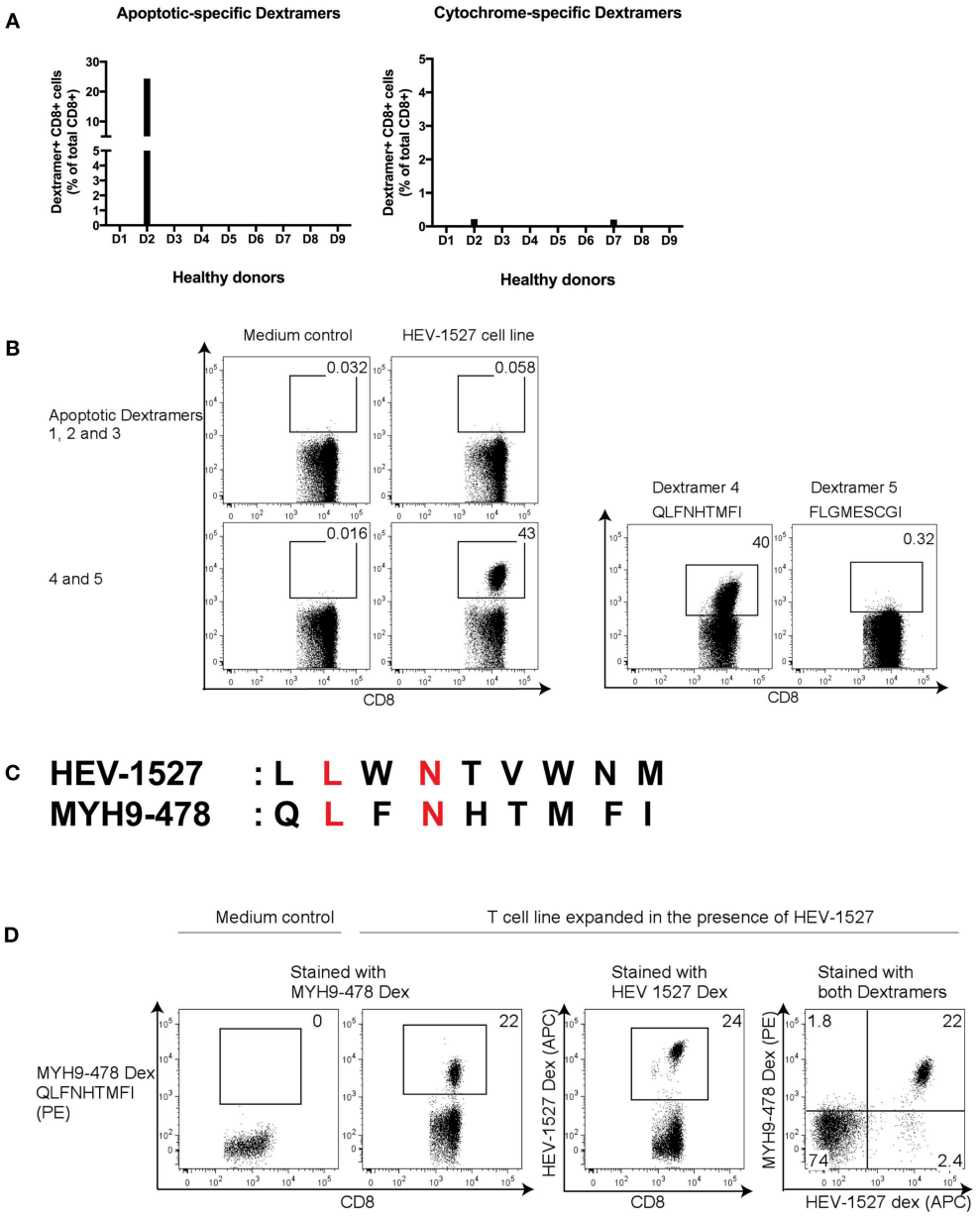

FIGURE 2 | Screening of potential cross-reactive self-antigens in HEV T cell lines. **(A)** CD8+ T cells from healthy donors (D1 to D9) were expanded in HEV-1527 peptide, followed by apoptotic- and cytochrome-specific Dextramers staining. Five Dextramers in each group were combined in a single staining. **(B)** Identification of target apoptotic antigen using Dextramers stained individually. **(C)** Amino acid sequence comparison between HEV-1527 and MYH9-478; matching amino acids were highlighted in red. **(D)** Double Dextramer staining of the two epitopes in HEV-1527-T cell line. FACS plots are gated on CD8+ T cells.

shows that constructs B- or D-redirected T cells, both equipped with TCRAV12-02, were able to bind both HEV-1527 and MYH9-478 Dextramers, demonstrating that TCRAV12-02 alone was responsible for the cross-recognition of these two peptides. The Dextramer staining of HEV-1527 was higher than that of MYH9-478, substantiating higher avidity of TCRAV12-02 toward HEV-1527.

However, T cells redirected with constructs A and C, both harboring TCRAV04-01, had specificity to neither epitopes. Moreover, when T cells were redirected with a mix of effective and non-effective constructs (i.e., in A+B or C+D), the Dextramer-binding capacity was reinstated.

More importantly, T cell function followed the same pattern as Dextramer staining. In **Figure 5B**, when engineered T cells were stimulated by HEV-1527 peptide-loaded T2 cells, only the T cells expressing TCRAV12-02 responded by cytokine production (in constructs B and D), but not the T cells expressing TCRAV04-01 (in constructs A and C). In addition, TCR-redirected T cells remained non-responsive to MYH9-478 peptide-loaded T2 cells, in line with the *in-vitro* observation in **Figure 3**.

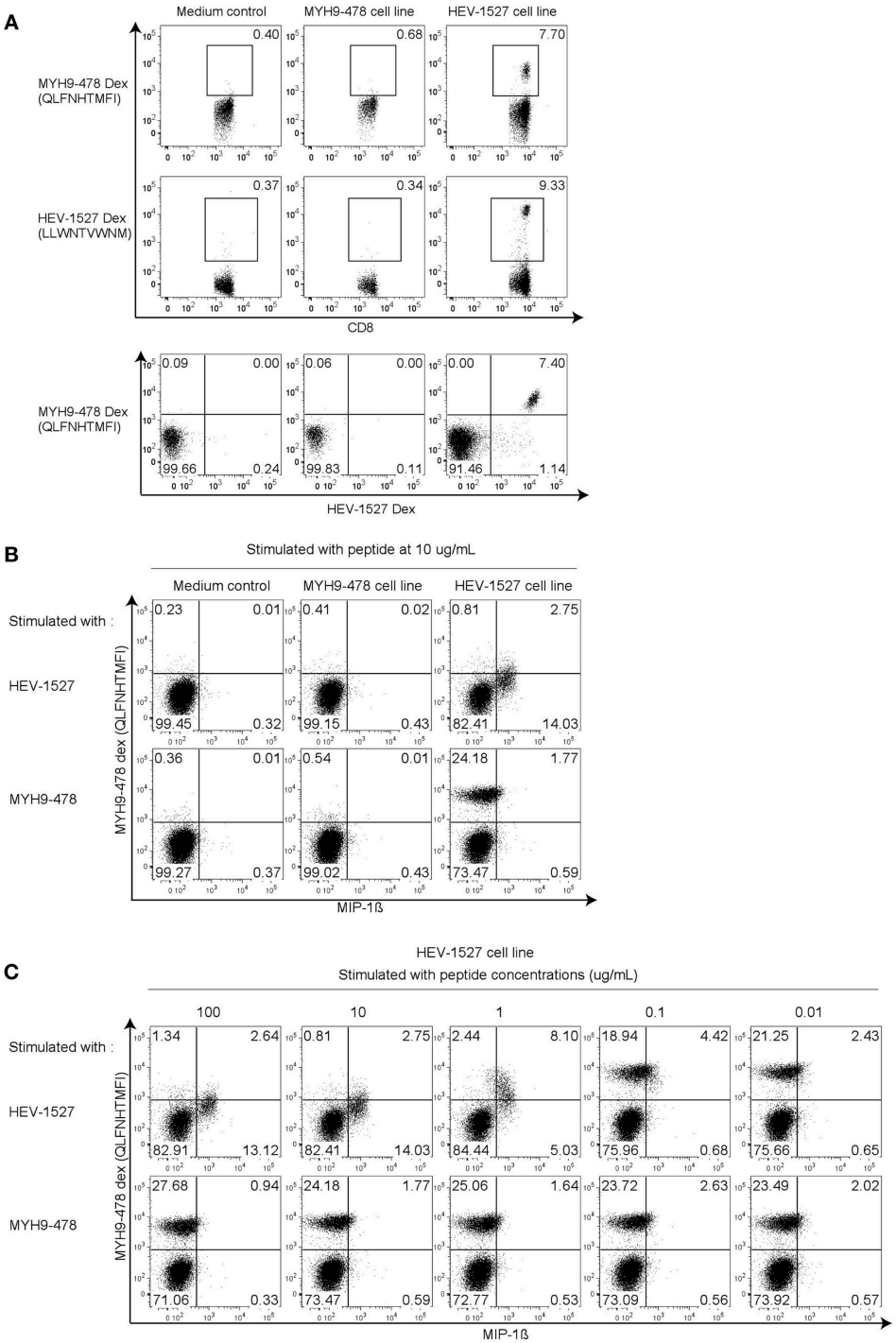

FIGURE 3 | Cross-reactivity is non-reciprocal. **(A)** Dextramer staining in T cell lines generated in the presence of MYH9-478 and HEV-1527 peptides. Double Dextramer staining was shown at the last row. **(B)** Functional intracellular cytokine staining in T cell lines generated from **(A)**, stimulated by respective peptides. **(C)** Functional assay in HEV-1527 cell line stimulated by different peptide concentrations. FACS plots are gated on CD8+ T cells.

Lastly, **Figure 5C** compared the polyfunctionality of engineered T cells upon stimulation by HEV-1527, using one effective and one non-effective construct as example (constructs A and B, respectively).

Through this assay, we discovered that the presence of either 4 or 5 glycines in TCRBV04-02 clonotype did not alter TCR specificity or function. Rather, it was TCRAV12-02 that make-or-break the fate of the TCR-mediated immunity.

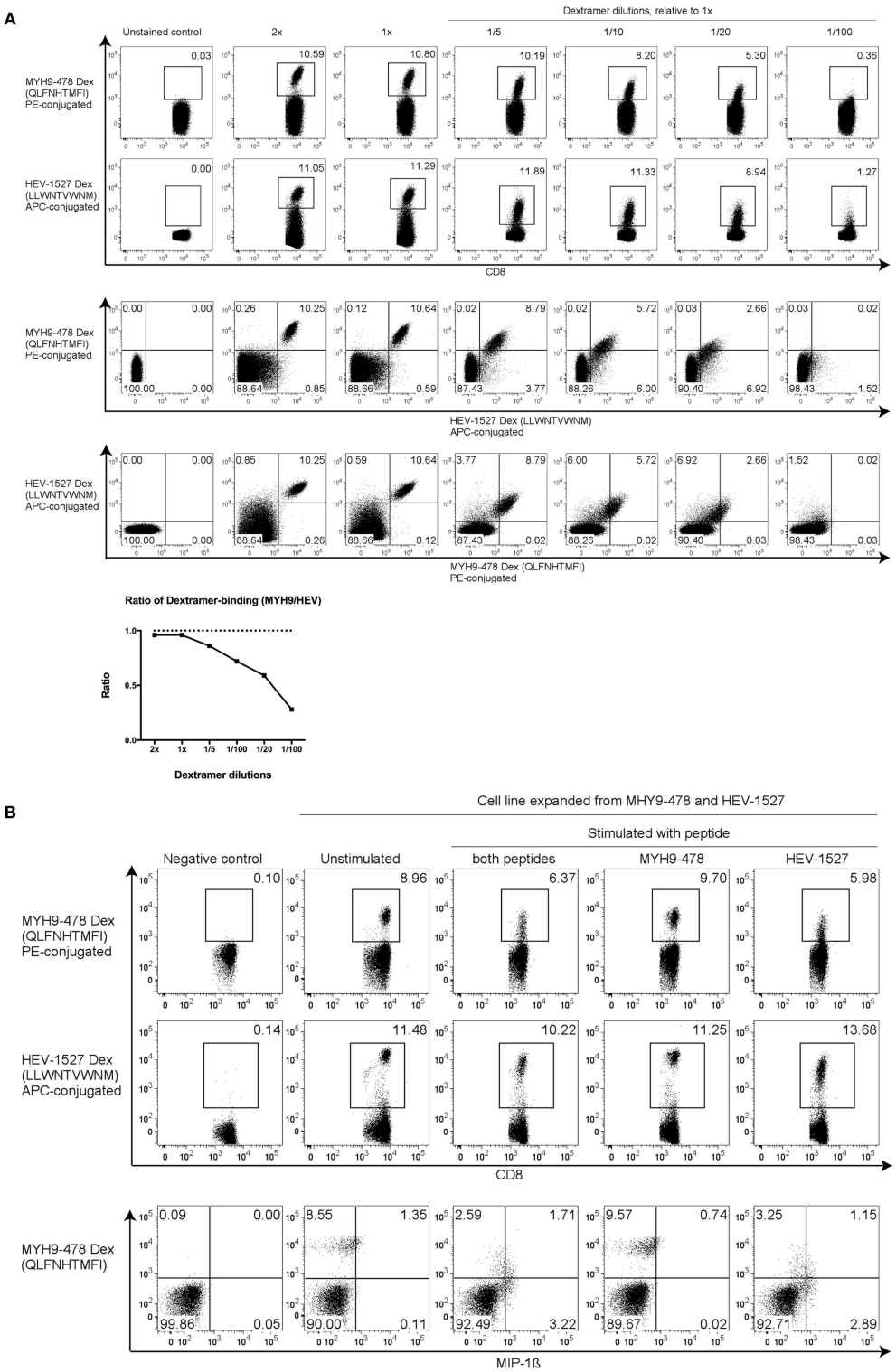

FIGURE 4 | Higher T cell avidity toward HEV peptide. **(A)** HEV-1527 and MYH9-478 Dextramer staining with dilutions to show T cell sensitivity. The recommended volume of Dextramer used for staining was denoted as 1x, the lower dilutions were adjusted accordingly by using FACS buffer as diluent. Double amount of Dextramer for the same number of cells was denoted as 2x. Dextramer staining was shown as either single- or double-gated, followed by a ratio of Dextramer-binding cells (MYH9/HEV). **(B)** Dextramer staining and functional assay with T cells expanded in the presence of both MYH9-478 and HEV-1527 peptides, stimulated by the peptides as indicated. FACS plots are gated on CD8+ T cells.

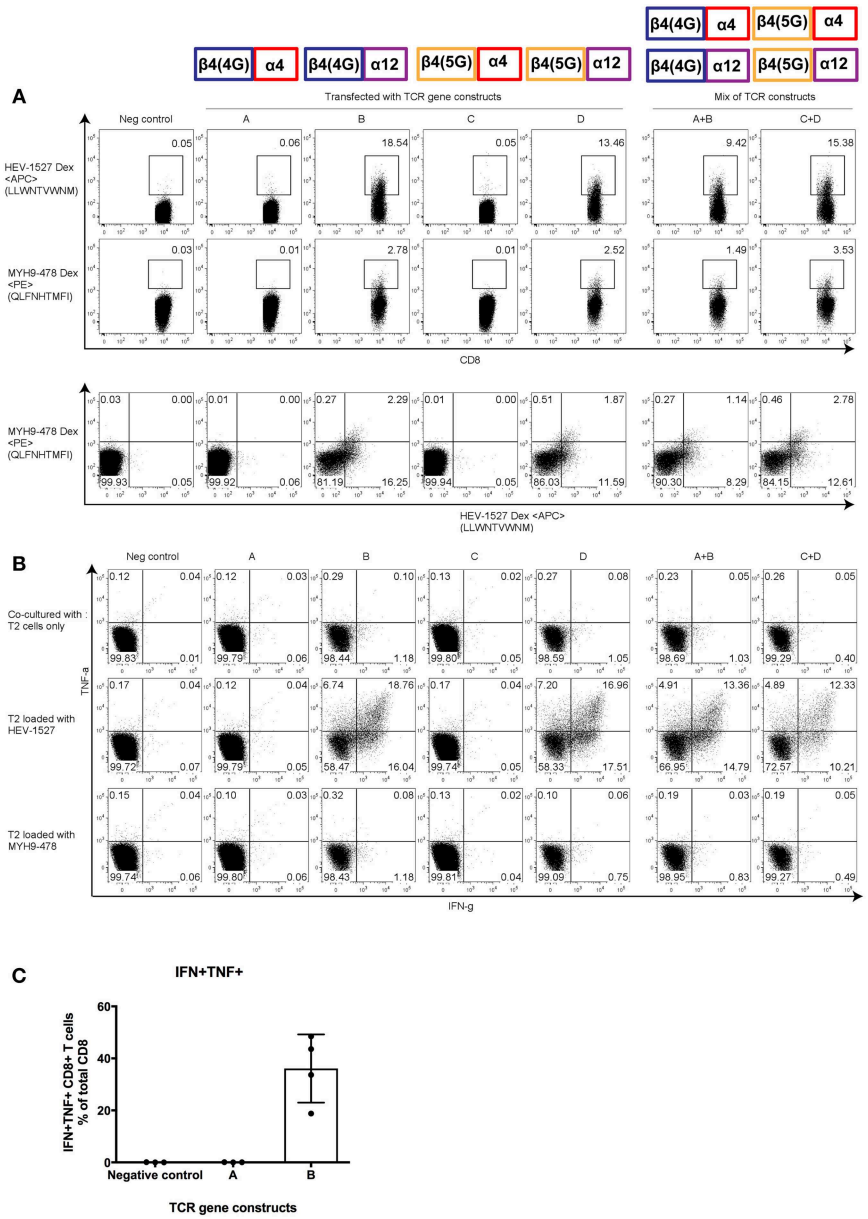

FIGURE 5 | Functional characterization of α and β clonotypes. Effector T cells were redirected with gene constructs encoding different TCRs, constructs A to D, to assess the effective α-β combination that constituted the **(A)** Dextramer-binding ability and **(B)** poly-functionality of T cells when stimulated by cognate peptide presented on the target T2 cells. Mixed constructs (A+B and C+D) were done by mixing the mRNA of two constructs prior to transfection. **(C)** Polyfunctionality of CD8+ T cells redirected with one effective and one non-effective construct (construct A and B, respectively). Results shown are mean ± SD; $n = 4$. Representative FACS plots are gated on CD8+ T cells; taken from one of four independent experiments. At the top of the figures are illustrations to show the α and β pairing of each gene constructs, as indicated in **Supplementary Figure 3**.

TCR β Chain With Multiple Glycines Could Facilitate Cross-Recognition of TCRAV12-02

We have established that TCRAV12-02 as the dominant α clonotype that was accountable for dual specificities and decreed TCR function, while TCRAV04-01 was silent (or specific against a peptide that is undetermined for now). By modeling the TCR-interacting surface, we could gain insight into the structural similarity between HEV-1527 and MYH9-478 peptides when presented by HLA-A*02:01 allele. As shown in **Figures 6A,B**, despite sharing only two out of nine amino acids, the two peptides present similar topographies and charge distributions when displayed by HLA-A*0201. Such similarity in physiochemical properties is observed in the amino-terminal portion of the peptide, which is contacted by the α chain of the TCR (left hand side of the structures, **Figure 6D**). This observation might explain

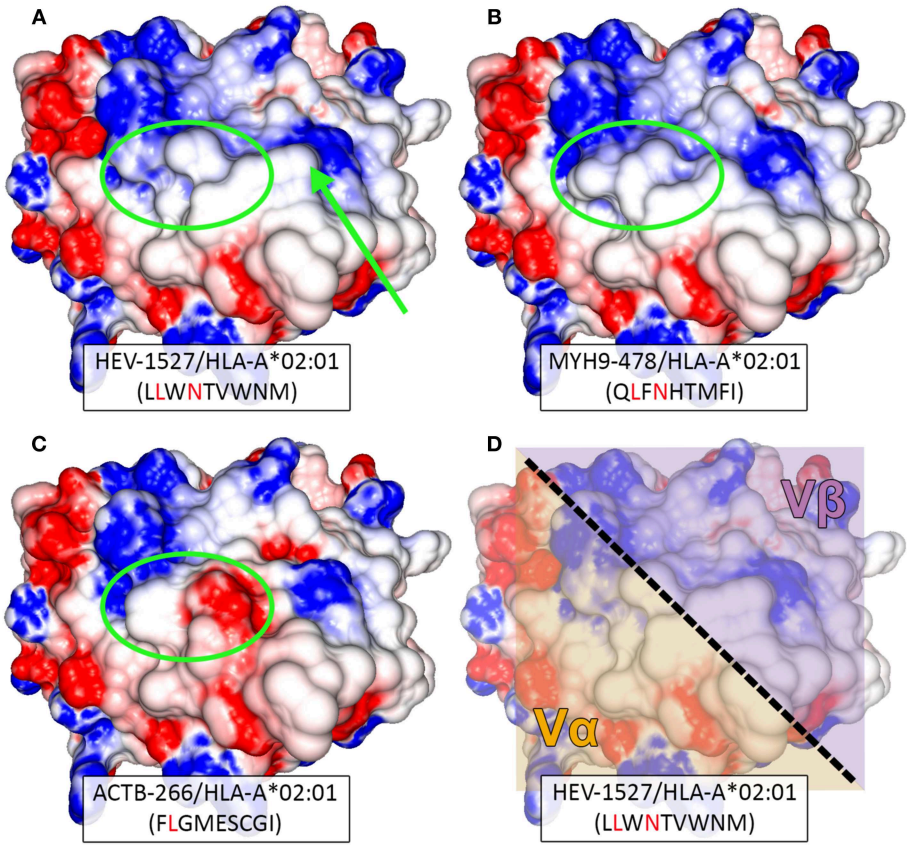

FIGURE 6 | Structural modeling of peptide-HLA complexes. Structure-based comparison between **(A)** HEV-1527, **(B)** MYH9-478, and **(C)** ACTB-266. Corresponding peptide sequences are depicted below each complex, with matching amino acids highlighted in red. Greater structural similarity is observed between HEV-1527 and MYH9-478, than with ACTB-266, especially in the amino-terminal portion of the peptide (green ellipsis). A potentially outstanding structural feature in the carboxy-terminal of HEV-1527, determined by a tryptophan at position 7, is indicated by an arrow. **(D)** The approximated area of interaction for the variable regions alpha (Vα) and beta (Vβ) of the TCR are depicted over the surface of the HEV-1527/HLA-A*02:01 complex.

why TCRAV12-02 could cross-recognize the two peptides. On the other hand, structural differences at the same region could prevent recognition by TCRAV12-02, as observed in the case of ACTB-266 peptide, which is one of the apoptotic epitopes that we screened (**Figure 6C** and **Supplementary Table 1**). In addition, there was a noticeable structural difference between HEV-1527 and MYH9-478 at P7 (the 7th amino acid of the epitopes, as indicated by an arrow), which is at the point of contact by TCR β chain.

Based on the evidence we had, it could be inferred that the binding promiscuity as a result of multiple glycine motif in TCR β chain might allow the TCR to overcome the structural impediment (at P7) to cross-recognize a secondary peptide complex.

DISCUSSION

In this study, we investigated a HEV-specific T cell receptor (TCR) which we proposed as a candidate in T cell-based therapy, to treat chronic hepatitis E. We focused on a TCR comprised of two α chains (TCRAV12-02 and TCRBV04-02) and one prevalent β chain containing a multiple glycine motif in the CDR3 region, to investigate its potential autoimmunity associated with TCR-based immunotherapy.

We started by screening plausible self-antigen peptides that this HEV-specific TCR might recognize, and discovering an apoptosis-related epitope that could be cross-recognized by the HEV-specific T cells. This pair of peptides, a HEV peptide and a self-antigen epitope (HEV-1527 and MYH9-478, respectively) shared only two matching amino acids, which suggests that molecular mimicry may not be the main mechanism behind such cross-reactivity. Other mechanisms of cross-reactivity could be the reason, such as the possession of dual α chains or alternative recognition (12, 28).

Our subsequent data showed that these cross-recognizing T cells had higher avidity toward the HEV peptide, which might help to explain why we did not observe a reciprocal cross-reactivity. When HEV-1527 peptide was used to expand the donor's T cells, this resulted in the proliferation of T cells with specificities for both HEV-1527 and MYH9-478. In contrast, exposure to MYH9-478 peptide did not result in the proliferation of MYH9-478-specific, nor HEV-1527-specific T

cells. Such non-reciprocal cross-reactivity was characterized in mice, where heterologous immunity was discovered (28). After lymphocytic choriomeningitis virus (LCMV) infection, mice were found to develop protective cross-reactive immunity against the subsequent vaccinia virus (VACV) challenge, but the reverse was not true. This could be explained by the private specificity of TCRs and the sequence of event/infection (29–31), which steered us to focus on the TCR repertoires.

To shed light on this, TCR redirection assays were used to confirm the cross-reactivity, through which we incidentally discovered that one α chain (TCRAV12-02) alone was accountable for dual specificities. Structural analysis of the complexes recognized by this TCR helped clarifying the molecular basis for this cross-recognition. First, despite aforementioned sequence dissimilarity between the two peptides, surprising structural similarity can be observed when analyzing the TCR-interacting surfaces of the peptide-HLA complexes. This structural similarity could allow both peptides being recognized by the same α chain (TCRAV12-02). The aromatic ring at position 7 of the HEV-1527 peptide could represent an outstanding feature that limits or prevents reciprocal cross-reactivity with MYH9-478 (32). This difference was observed at the carboxy-terminal portion of the peptide, within the region of contact for TCR-β CDR3. We speculated that the presence of multiple glycines in the β chain may render the TCR to be more flexible when docking peptide-HLA-A2 complex (15), because conformational changes occur more readily in CDR3 than in CDR1 or CDR2 (33). Since glycine is smallest in size and neutral in charge, a structural change is easily achieved with minimal energy threshold. This flexibility in β chain may partially compensate for the structural difference, contributing to cross-recognition.

Cross-reactive T cells are canonically associated with auto-reactive phenotypes. However, the TCR that we reported here only recognize but not react toward the self-antigen (MYH9-478). Similar observation was chronicled in mice, where cross-reactive T cells did not respond the same way to each of the two target peptides (30), it is hence logical to deduce that TCR may display preferential affinity to one favored target over the other.

There are limitations in our study which we would like to highlight, such as the exclusive use of T2 cells sensitized with peptide as target cells in functional assays. Using a co-culture system with TCR-redirected T cells and a HEV-infected hepatocyte cell line would allow the analysis of TCR responses to naturally processed and presented HEV epitopes. We also cannot fully rule out that a functional response to the self-peptide may be possible under inflammatory conditions or that other cytokines are stimulated, rather than IFN-γ, TNF-α, or MIP-1β. Although, it has been suggested that MIP-1β is very sensitive to detect cross-reactive T cell responses (34).

In addition, although we included only a limited scope of self-antigens in the *in-vitro* screening to detect TCR auto-reactivity, we prioritized the selection to emphasize on those with high likelihood and relevance to clinical manifestation. Nonetheless, it is still prudent to test it in *in-vivo* models, such a humanized mouse model developed for HEV studies (35–37), before advancing it further for immunotherapy.

Based on our results, we suggest that screening of self-antigens should be an important undertaking to be incorporated into the developmental phase of redirected TCR therapies. Although our selection of self-antigens was non-exhaustive, we included additional analysis, specifically by modeling the peptide-HLA complex (32). This method has the potential to predict cross-reactivity based on structural similarity, rather than peptide sequence identity alone. Such innovative way of interpreting cross-reactivity is particularly suitable for peptides of low amino acid similarity, as further evidenced by our results (**Figure 2C**).

In summary, TCRs in possession of the hallmarks of cross-reactivity such as multiple glycines should be carefully assessed in the design of immunotherapies in order to minimize off-target toxicity. Nevertheless, the candidate TCR in our case did not show functional cross-reactivity. **Figure 7** gives a graphical summary of our findings on this HEV-specific TCR.

MATERIALS AND METHODS

Study Cohorts

This study was reviewed and approved by the ethic committee of Hannover Medical School, approval number 2315-2014. All healthy donors ($n = 9$) were recruited in Hannover Medical School for a previous study, in which their HEV seroprevalence were tested negative (11). HLA phenotyping on all donors was done by antibody staining (mouse anti-human HLA-A2, clone BB7.2, Alexa-Fluor 647; Bio-Rad Laboratories, USA); all individuals were HLA-A2 positive. Written informed consents for participating in this research study and blood draws were collected from all individuals.

Sequence-Specific Peptides and Dextramers

All peptides used in the study were synthesized by ProImmune, UK. Peptide sequences specific to HEV are of genotype 3, based on data from GenBank accession number AF455784. Peptides were dissolved in Dimethyl sulfoxide (DMSO) to yield stock solutions of 60 mg/mL, which were further diluted with HBSS to be used in cell culture.

Dextramers bearing the selected epitopes were synthesized by Immudex, Denmark. All Dextramers were specific for HLA-A*02:01 allele, and conjugated with PE fluorochrome (except HEV-1527 Dextramer, which was conjugated with APC fluorochrome). In Dextramer dilution experiment, various dilutions were prepared by using FACS buffer as diluent to yield the dilution factors as indicated in **Figure 4**.

CD8+ T Cell Culture (3-Week T Cell Expansion)

CD8+ T cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy donors using magnetic CD8+ T cell isolation beads according to protocol (Miltenyi Biotec, USA). Once isolated, cells were kept in AIM-V medium supplemented with sodium pyruvate, non-essential amino acids, 5 mM HEPES buffer, 0.5% β-mercaptoethanol (all from Gibco, Life technologies), 10% human AB serum (PAN Biotech GmbH,

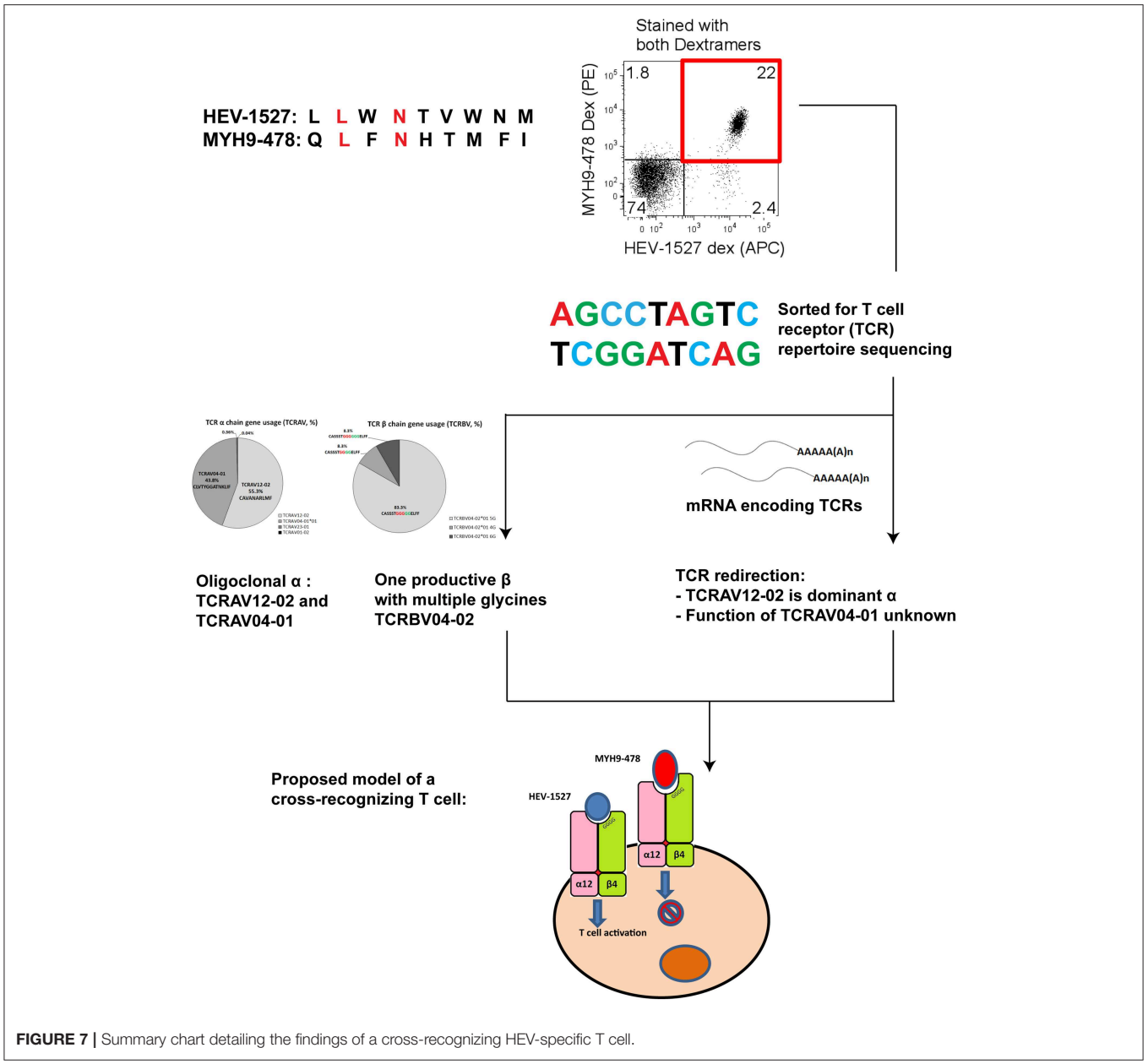

FIGURE 7 | Summary chart detailing the findings of a cross-recognizing HEV-specific T cell.

Germany), and 5 IU/mL IL-2. Peptide-loaded T2 cells (ATCC CRL-1992) were irradiated before co-cultured with CD8+ T cells at a ratio of 1:5 (1 T2 cell: 5 T cells). Peptide loading concentration was 1 μg/mL. The cell culture had a change of media every 3–4 days, and irradiated peptide-loaded T2 cells were replenished every week. The entire culture duration lasted 3 weeks.

T Cell Proliferation and Functional Assays

Post-expansion, proliferation of self-antigen-specific T cells was detected by Dextramer staining. Dextramers bearing predicted epitopes of caspase-cleaved products associated with apoptosis or epitopes related to autoimmune hepatitis are summarized in **Supplementary Table 1**. All five Dextramers per group were combined in a single staining. Once positive staining was identified, the cells were stained with individual Dextramer to single out the target epitope.

T cell functional assay was assessed by intracellular cytokine staining; 0.2×10^6 T cells were plated, to which peptides for stimulation were added (concentrations as indicated in text), in the presence of Brefeldin A at 2 μg/mL for 6 h incubation. Thereafter, cells were washed with FACS buffer and stained with Dextramer for 20 min at room temperature. Then, staining of surface markers for 10 min at room temperature (BD Bioscience: FITC anti-CD14 clone M5E2 and anti-CD19 clone HIB19, fixable green live/dead cell staining dye (Life technologies) for exclusion of monocytes, B cells and dead cells, respectively; and APC-H7 anti-CD8 clone SK1). Next, cells were fixed by fixation and permeabilization buffer for

20 min at 4°C and washed twice with perm/wash buffer (both from BD Bioscience). Staining with antibodies for intracellular cytokines was performed for 30 min at 4°C in the dark (PE-Cy7 anti-MIP-1β clone D21-1351 and BV-421 anti-TNF-α clone Mab11 from BD Bioscience, and BV-711 anti-IFN-γ clone 4S.B3 from BioLegend, USA). Cells were washed twice and acquired using BD LSR Fortessa flow cytometer and analyzed by FlowJo version 9. Gating strategy is outlined in **Supplementary Figure 1**.

CD8+ T Cell Receptor Repertoire Sequencing

Dextramer-specific CD8+ T cells were sorted using Dextramers, and shipped to Adaptive Biotechnologies (Seattle, USA) for sequencing of complimentary determining region 3 (CDR3) of both α and β chains (ImmunoSEQ) by Next Generation Sequencing, as described (11).

Sanger Sequencing was performed using the Dextramer-sorted cells to confirm the results from Next Generation Sequencing independently. Total RNA was isolated from the sorted cells using Qiagen RNeasy Plus Micro Kit (Qiagen, Germany). Then, cDNA was transcribed from the total RNA using SMARTer PCR cDNA Synthesis kit (Clontech Laboratories), as described (38). PCR was performed using Advantage 2 PCR Kit (Clontech Laboratories) to amplify the genes of T cell receptor, with primers targeting constant regions of α and β chains:

α chain primer: 5′-GGAACTTTCTGGGCTGGG GAAGAAGGTGTCTTCTGG-3′

β chain primer: 5′-TGCTTCTGATGGCTCAAACACA GCGACCT-3′

The cycle conditions for PCR were: 1 cycle of 30 s at 95°C, 5 cycles of 5 s at 95°C and 2 min at 72°C, 5 cycles of 5 s at 95°C and 10 s at 70°C and 2 min at 72°C, 35 cycles of 5 s at 95°C, and 30 s at 68°C and 2 min at 72°C, lastly 1 cycle at 4°C. PCR products of both TCR chains were gel-purified using MinElute Gel Extraction Kit (Qiagen, Germany) and cloned into pCR4-TOPO vector using TOPO TA Cloning Kit for Sequencing (Invitrogen). Cloned plasmids were transformed into TOP10 chemically competent *E.coli* (Invitrogen), and plated on LB agar plates supplemented with ampicillin overnight for colony growth at 37°C incubator. Colonies were picked the next day and sent for Sanger Sequencing (GATC). Sequencing results of CDR3 regions were aligned with IMGT database (ImMunoGeneTics, http://www.imgt.org).

The presence of a dominant β and its number of multiple glycines were also further verified by Deep Sequencing, as detailed elsewhere (39). In short, RNA was transcribed into cDNA using SMARTer PCR cDNA Synthesis kit (Clontech Laboratories) followed by amplification of the CDR3 region of β chain only, using Advantage 2 PCR Kit (Clontech Laboratories). Purified amplicons were then subject to sequencing on the Illumina MiSeq platform using a 600 cycle v3 MiSeq Reagent Kit. The sequencing output was annotated by the IMGT database. Productive reads were then subject to further bioinformatics analyses using tcR R-package and VDJtools as published (40, 41).

T Cell Receptor (TCR) Constructs Design

Nucleotide sequences from Next Generation Sequencing were used to design TCR constructs. Codon optimization and murinized constant chains were adopted (27, 42), to avoid target αβ-TCR mispairing with endogenous TCRs expressed by the recipient T cells. Genes of TCR constructs were cloned into *E.coli* for amplification. Plasmid DNA containing the TCR genes were purified and linearized with XbaI restriction enzyme (Thermo Fischer Scientific) (27). Thereafter, mRNA was synthesized from linearized DNA using mMESSAGE mMACHINE T7 Ultra Kit (Life technologies), for TCR redirection.

T Cell Receptor (TCR) Redirection Assay

PBMCs of healthy donor were expanded for 7 days in AIM-V medium supplemented with 2% human AB serum, 600 IU/mL IL-2 and 50 ng/mL anti-CD3 (clone OKT3, BioLegend, USA). One day before redirection, IL-2 concentration was adjusted to 1,000 IU/mL (27). For each TCR gene construct, 10×10^6 cells were resuspended in Nucleofector solution, to which 20 μg of mRNA was added, and transferred into a cuvette for electroporation, using Amaxa Cell Line Nucleofector Kit V and Lonza Nucleofector 2b device (program X-01). After electroporation, redirected cells were kept in AIM-V medium supplemented with 2% human AB serum and 100 IU/mL IL-2. For mixed construct transfection (A+B or C+D), the mRNA of both constructs were mixed, then handled as described above. In T cell stimulation assay, peptide-loaded T2 cells were co-cultured with engineered-T cells at an effector: target cell ratio (E:T ratio) of 1:1. Peptide loading concentration in T2 cells was 1 μg/mL. The functional cytokines were assessed through intracellular cytokine staining as outlined above.

Peptide-HLA Structural Modeling and Analysis

The 3D structures of the peptide-HLA complexes of interest were predicted using an in-house implementation of DockTope (43). Briefly, a reference crystal structure of HLA-A*0201 was used as the receptor for a molecular docking with the modeled peptide-ligand. The docking search was performed with Autodock Vina 1.1.2 (44), followed by full atom energy minimization with Gromacs 4.6.5 (45), and a second docking search with Vina (43, 46). Electrostatic potential over the TCR-interacting surface of modeled complexes was calculated using Delphi (47), and top-view images were generated through the molecular viewer software GRASP2 (48).

DATA AVAILABILITY

GenBank accession number of hepatitis E virus (HEV) sequences used in peptide synthesis is AF455784, as mentioned under Materials and Methods section.

AUTHOR CONTRIBUTIONS

CS, SZ, PS, HW, MS, and MC contributed to study design and experiments. CS acquired and analyzed data. SZ

provided technical guidance. PS applied for DFG grant funding. MM recruited human study subjects. CS-F and SR performed deep sequencing experiments and analyzed the data. DA performed the structural modeling and analyzed the data. CS and MC drafted and revised the manuscript. All authors read and agreed the manuscript. MC approved the finalized 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.2019.02076/full#supplementary-material

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Enhancing Immune Response and Heterosubtypic Protection Ability of Inactivated H7N9 Vaccine by Using STING Agonist as a Mucosal Adjuvant

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Influenza vaccines for H7N9 subtype have shown low immunogenicity in human clinical trials. Using novel adjuvants might represent the optimal available option in vaccine development. In this study, we demonstrated that the using of the STING agonist cGAMP as a mucosal adjuvant is effective in enhancing humoral, cellular and mucosal immune responses of whole virus, inactivated H7N9 vaccine in mice. A single dose of immunization was able to completely protect mice against a high lethal doses of homologous virus challenge with an significant dose-sparing effect. We also found that intranasal co-administration of H7N9 vaccine with cGAMP could provide effective cross protection against H1N1, H3N2, and H9N2 influenza virus. Furthermore, cGAMP induced significantly higher nucleoprotein specific $CD4^+$ and $CD8^+$ T cells responses in immunized mice, as well as upregulated the IFN-γ and Granzyme B expression in the lung tissue of mice in the early stages post a heterosubtypic virus challenge. These results indicated that STING agonist cGAMP was expected to be an effective mucosal immune adjuvant for pre-pandemic vaccines such as H7N9 vaccines, and the cGAMP combined nasal inactivated influenza vaccine will also be a promising strategy for development of broad-spectrum influenza vaccines.

Keywords: H7N9, whole viron vaccine, STING, mucosal adjuvant, cross protection

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INTRODUCTION

In March 2013, the first identified case of human infection with avian influenza A (H7N9) virus occurred in China, and as of 5 September 2018, a total of 1,567 human infections with H7N9 viruses, including at least 623 deaths, were reported during the fifth epidemic wave (1, 2). More importantly, some novel biological features of the H7N9 virus, such as the high frequency of drug-resistance, emergence of highly pathogenic outbreaks in chickens and humans were discovered in this recent fifth epidemic wave in 2017 (2–4). According to the result of the United States CDC's Influenza Risk Assessment, the avian influenza A(H7N9) virus is now ranked as the influenza virus with the highest potential pandemic risk among all influenza viruses (5).The continuous evolution of the H7N9 virus poses a long-term threat to public health, and thus it is imperative to strengthen prevention and control strategies.

Vaccination is the most effective way to prevent against seasonal and pandemic influenza caused by influenza viruses. Since the first outbreak of H7N9, different types of candidate H7N9 vaccines have been developed and are currently undergoing clinical trials. However, published clinical data demonstrated that H7N9 vaccines show poor immunogenicity in humans and using of novel adjuvants, such as MF59, AS03, immuno-stimulating complex (ISCOM), and aluminum hydroxide may have an important effect on improving vaccine immunogenicity for the uniquely low immunogenicity of this strain (6–9).

The respiratory tract mucosa is the site of infection for influenza viruses and the local immune responses on mucosal surfaces play an important role in defense against viral infection (10). Several studies revealed that the intranasal administration of inactivated vaccines, combined with an appropriate adjuvant induced well protection and cross protection against infection by both homologous and heterosubtypic viruses (11–13).

Cyclic GMP-AMP (cGAMP) is an endogenous cyclic dinucleotide catalysts synthesized by the recently discovered cyclic-GMP-AMP synthase (cGAS), which was activated by pathogen-derived cytosolic double stranded DNA. The cGAMP can be bound to the stimulator of interferon genes (STING), leading to the activation of IRF3 and induction of interferon-β, thus cGAMP functions as an endogenous second messenger in innate immune signaling by cytosolic DNA (14, 15). Both *in vivo* and *in vitro* studies suggest that cGAMP could be used as an effective adjuvant for a model antigen, like OVA and vaccines, such as porcine reproductive and respiratory syndrome virus (PRRSV) virus-like particles, and anthrax toxins (16–18). Recently, cGAMP have also been demonstrated to be an ideal adjuvant for cutaneous vaccination of influenza vaccine (19).

Besides adjuvant effect, safety issues for the cGAMP have to be considered. cGAMP is a natural metabolizable molecule in humans and is hydrolyzed quickly by ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1) when located outside the plasma membrane, ensuring that its adjuvant activity is transient, effectively circumventing unwanted systemic inflammation (16). In addition, studies have shown that cGAMP does not cause any significant skin or acute local inflammatory responses and is not toxic to the liver or kidney (18, 19). Therefore, as a natural ligand for STING, cGAMP might be a more promising candidate adjuvant for next generation vaccines.

In terms of convenience of vaccination and the capability of inducing cross protection by mucosal immunization, a mammalian 2′, 3′-cGAMP was used as a mucosal adjuvant for inactivated whole-virion H7N9 influenza vaccine in the present study. We demonstrated that cGAMP enhances serum and mucosal antibodies, T cells, innate immune responses, as well as the protective ability of H7N9 vaccine in mice. Further, we showed that intranasal delivery of inactivated H7N9 vaccine formulated with cGAMP can induce a more robust T cell response against virus conserved epitopes that mediate cross protection against heterosubtypic influenza A viruses. Therefore, the cGAMP may be a promising vaccine adjuvant for the broad-spectrum influenza vaccines.

MATERIALS AND METHODS

Vaccine, Viruses, Mice, and Adjuvants

An egg-derived, formalin-inactivated whole-virion H7N9 influenza vaccine based on vaccine candidate virus A/Shanghai/2/2013 H7N9 (NIBRG-267) was manufactured by Shanghai Institute of Biological Products (**Figure S1**). The vaccine has passed the quality control test in accordance with the requirements of Chinese Pharmacopeia (2015, Edition 3), and now is currently under phase II clinical trials. Influenza viruses used in this study included mouse adapted A/Shanghai/2/2013 (Sh2/H7N9), A/PR/8/34 (H1N1) virus, A/Guizhou/54/1989 (Gz54/H3N2), and A/Chicken/Jiangsu/7/2002 (H9N2) viruses as described in our previous studies (20, 21). Specific pathogen free (SPF) female BALB/c mice (6–8 weeks old) were purchased from Shanghai Laboratory Animal Center, China. All mice were bred in the animal resource center at Shanghai Institute of Biological Products and maintained under SPF conditions with constant temperature and humidity. The protocol for the animal study (Protocol Number: 17-1250) was approved by the laboratory animal management committee, and the laboratory animal ethics and welfare protection group of Shanghai Institute of Biological Products. All animal procedures were carried out in accordance with the animal ethics guidelines of the Chinese National Health and Medical Research Council (NHMRC). Adjuvant 2′-3′-cGAMP (Invivogen) was diluted with endotoxin-free water to a concentration of 1 mg/mL.

Immunization and Viral Challenge

For a homologous protection study, mice were intranasally immunized once with different doses (0.015 ug, 0.15 ug, and 1.5 ug HA) of H7N9 vaccine alone or with 5 ug 2′-3′-cGAMP in a total volume of 25 ul. The 5 ug 2′-3′-cGAMP immunized and an unimmunized group was used as an adjuvant control group and negative control group respectively. Three weeks after immunization, mice were anesthetized and challenged intranasally with 20 μl of the viral suspension containing $40\times LD_{50}$ of A/Shanghai/2/2013 (Sh2/H7N9) virus. For a heterosubtypic protection study, mice were intranasally immunized with either 1.5 ug HA of H7N9 vaccine alone or with 5 ug 2′-3′-cGAMP twice on day 0 and 21. Three or six weeks (for long term protection) after the last immunization mice were anesthetized and challenged intranasally with 20 μl of the viral suspension containing $5\times LD_{50}$ of A/PR/8/34 (H1N1) or A/Guizhou/54/1989 (Gz54/H3N2) or A/Chicken/Jiangsu/7/2002 (H9N2) influenza virus. Survival and body weight loss were monitored for 2 weeks post virus challenge.

Specimens Preparation

Five mice from each group were randomly chosen for sample collection at a predetermined time after immunization or virus challenge (see Results). The sera were collected from the blood and used for serum IgG, Hemagglutination inhibition (HI) antibodies assays. The spleens were taken out by sterile forceps to prepare PBMC. Mouse lungs were collected and homogenized in 1.5 mL of PBS containing Penicillin-Streptomycin (Gibco, USA) by an electric homogenizer Tissuelyser-24 (Jingxin, Shanghai,

China). Finally, a syringe needle with 1 mL of PBS was inserted three times into the nasopharynx to collect the nasal wash. The lung homogenates and nasal wash were centrifuged to remove cellular debris.

Antibody Assays

The titers of virus specific IgG and IgA of mice 3 weeks after a single dose immunization were measured by enzyme-linked immunosorbent assay (ELISA), which was performed using a series of reagents consisting of: firstly, 5 μg/mL of inactivated whole-virion H7N9 vaccine for plate coating; secondly, serial 2-fold dilutions of sera or nasal wash or lung homogenates; thirdly, goat anti-mouse IgG Ab (γ-chain specific) (KPL) or goat anti-mouse IgA (α-chain specific) (KPL) conjugated with horseradish peroxidase (HRP); and finally, the substrate 3, 3′, 5, 5′-Tetramethylbenzidine (TMB). The amount of chromogen produced was measured based on absorbance at 450 nm. Ab-positive cut-off values were set as means+2×SD of PBS control group. An ELISA Ab titer was expressed as the highest serum dilution giving a positive reaction.

The hemagglutination inhibition (HAI) antibody titers of sera against different virus strains by a one or two doses immunization was determined by HI assay. Briefly, sera were pretreated with a receptor destroying enzyme (Diho, China) for 20 h at 37°C and then inactivated at 56°C for 30 min; 2-fold serial dilutions of 50 μl pretreated sera and positive control sera were incubated with an equal volume of 4 HA units of selected virus antigen for 1 h at room temperature and then 50 μl of a 1% suspension of chicken red blood cells (RBC) were added. After 30 min of incubation at room temperature, the HI titers were determine by the highest dilution of sera that completely inhibits the agglutination of the chicken RBC. The limit of detection for this assay is a 1:10 dilution.

ELISpot Assays

Virus or nucleoprotein (NP) specific IFN-γ secreting splenocytes of immunized mice was determined by ELISpot assay as described in our previous study (20). For detecting virus specific IFN-γ secreting splenocytes, 10 μg/mL of H7N9 influenza vaccine was used as a stimulant. As well, an H-2d-restricted NP class I peptide and a pool of three H-2d restricted class II peptides as described in our previous study were used as stimulatory agents for detection of NP specific IFN-γ secreting $CD8^+$ T cells and $CD4^+$ T cells, respectively. The number of virus or peptide-reactive cells was represented as spot forming cells per 10^6 splenocytes and was calculated by subtracting spot numbers in control peptide (HIV pol peptide ILKEPVHGV) wells from that in NP specific peptide (or H7N9 influenza vaccine) containing wells. The number of peptide-reactive cells was represented as spot forming cells per 10^6 splenocytes.

Analyses of Lung Cytokines and Cytotoxic Effector Molecules

Lung homogenates from five mice in each group were collected at 24 h post a single dose immunization for detection of cytokines (IL-6, TNF-α, IL-1β) by mouse cytokine MILLIPLEX® MAP kits (MCYTOMAG-70K, Millipore) according to the manufacturer's protocol. Measurements were performed using the Bio-Plex MAGPIX Multiplex reader. Concentrations of IFN-γ and Granzyme B in whole lung homogenates of mice post a heterosubtypic influenza A viruses challenge were determined by quantikine mouse IFN-γ ELISA Kit (88-8314-22, invitrogen) and Mouse Granzyme B ELISA Kit (GWB-SKR178,GENWAY) according to the manufacturer's protocol.

Virus Titrations

Virus titration was performed as described previously (21). Lung homogenates were serially diluted 10-fold and loaded on confluent MDCK cells, which were subsequently incubated in the growth medium and tested for hemagglutination 72 h later. The virus titer of each specimen, expressed as the 50% tissue culture infection dose ($TCID_{50}$), was calculated by the Reed-Muench method.

Statistics

GraphPad Prism 5 software was used to perform statistical analyses. The survival rates of the mice in the test and control groups were evaluated by Log-rank (Mantel-Cox) test; the results of serum and mucosal antibody titers, lung virus titers and cytokine response were evaluated by one-way ANOVA and Tukey's multiple comparison test; if the p-value was < 0.05, the difference was considered significant.

RESULTS

cGAMP Adjuvanted Vaccine Offers Improved Protection Against a High Lethal Dose Challenge of Homologous H7N9 Virus

One twenty mice were randomized into 8 groups (A-H), with 15 mice in each group. Mice were all immunized intranasally (i.n.) once with various doses of whole-virion H7N9 influenza vaccine alone or in combination with cGAMP as an adjuvant, respectively; The control group was not immunized. All mice were then i.n. challenged with a high lethal dose ($40\times LD_{50}$) of mouse adapted A/Shanghai/2/2013 (Sh2/H7N9) viral suspension 3 weeks post-immunization. The lung hemogenate of five mice in each group was prepared and used for virus titration on day 3 after challenge. The survival rates and the body weight losses of the remaining 10 mice in each group were monitored for 2 weeks after the challenge.

The results presented in **Table 1** showed that the mice in the control group and the group immunized with cGAMP alone suffered a rapid reduction in body weight post virus challenge, and failed to provide any protection. The protection efficiency offered by H7N9 influenza vaccine alone was dependent on vaccine dosage, only the high dosage group could provide full protection against a high lethal dose challenge of influenza H7N9 virus, while the low and medium dosage groups provided 30 and 70% protection, respectively. In contrast, all the adjuvanted vaccine immunized groups could provide effective protection, regardless of the dosage (**Figure 1A**). Moreover, these mice showed relatively mild weight loss and faster recovery times following the challenge, as compared to the groups without cGAMP (**Figure 1B**).

TABLE 1 | Protection against a high lethal dose challenge of homologous influenza virus in mice by intranasal administration of whole-virion H7N9 influenza vaccine with or without cGAMP.

Group	Dose and adjuvant	Lung virus titer[a] ($log_{10}TCID_{50}$/ml)	Survival rate (No. of survivors/no. tested)
A	1.5μg + cGAMP	Undetected[b,c]	10/10[b]
B	1.5μg	Undetected[b]	10/10[b]
C	0.15μg + cGAMP	5.15 ± 0.42[b,c]	10/10[b]
D	0.15μg	7.60 ± 0.82[b]	7/10[b]
E	0.015μg + cGAMP	8.10 ± 0.93[b,c]	10/10[b,c]
F	0.015μg	11.60 ± 0.55	3/10
G	cGAMP	12.70 ± 0.62	0/10
H	control	12.45 ± 0.65	0/10

Mice were intranasally immunized once with various doses of whole-virion H7N9 influenza vaccine with or without cGAMP. Three weeks after the last immunization, mice were challenged with a high lethal dose (40×LD_{50}) of mouse adapted A/Shanghai/2/2013 (Sh2/H7N9) virus. Lung hemogenate from 5 mice in each group were collected 3 days post-infection for titration of lung virus. The survival rates of mice 2 weeks post-infection were determined.

[a] Results are expressed as mean ± SD of five tested mice in each group.

[b] Displays significant difference compared with mice in control groups ($P < 0.05$).

[c] Displays significant difference compared with mice in the corresponding non- adjuvanted groups ($P < 0.05$).

The results of lung virus titers were also shown in **Table 1**. The lung virus titer of immunized mice had a dosage dependent decreasing trend. Furthermore, the virus titer in cGAMP adjuvanted group was significantly lower than that of the non-adjuvanted group within the same dosage ($P < 0.05$). The obtained results indicate that a single dose immunization with inactivated whole-virion H7N9 vaccine plus cGAMP provided increased protection over vaccine alone, and reduced the viral load in the lungs after a high lethal dose challenge of a homologous H7N9 influenza virus.

cGAMP Adjuvant Enhances the Mucosal and Systemic Antibody and T Cell Responses of Whole-Virion H7N9 Influenza Vaccine

Forty mice were randomized into 8 groups, with 5 mice in each group, mice were immunized as described above. The titers of virus specific IgG and hemagglutination inhibition(HAI) antibodies in serum, virus specific IgA in nasal wash and lung hemogenate were detected at week 3 after immunization. The ELISpot was conducted to detect the cellular immune based on the amount of IFN-γ secreting splenocytes of immunized mice after being stimulated with whole-virion H7N9 influenza vaccine *in vitro*.

As shown in **Table 2**, all groups except the cGAMP group and the control group had an obvious serum antibody response in a dose-dependent manner, among which the serum antibody responses induced by the vaccine plus cGAMP were significantly higher than those in the vaccine alone group with the same dosage ($P < 0.05$). We also evaluated the serum HAI antibody titers, which have been correlated with the protective efficacy of influenza vaccines. The HAI antibody titers against the H7N9 virus elicited by the cGAMP adjuvanted vaccine were remarkably higher than those elicited by the vaccine alone. These results indicated that cGAMP was able to enhance the virus-specific antibody as well as the HAI antibody responses in mouse serum induced by whole-virion H7N9 influenza vaccine.

Since the influenza virus enters the body through the respiratory tract, determining the presence of antibodies in secretory mucosal samples is important. As also shown in the **Table 2**, although the IgA antibody in the nasal wash and lung hemogenate was undetected in the low dose of 0.015 μg in both adjuvant and non-adjuvanted groups, the medium and high dosage of the vaccine supplemented with cGAMP could induce high levels of mucosal IgA, which were significantly higher than those in the same dosage of non-adjuvanted groups ($P < 0.05$), indicating cGAMP can play a role as an effective adjuvant for inducing enhanced mucosal virus specific antibody responses against influenza virus.

The T cell response was analyzed by an IFN-γ ELISpot assay, and the results are shown in **Figure 2**. A trend toward increased IFN-γ secreting was noted in mice i.n. immunized with non-adjuvanted H7N9 compared with the control, however, this trend was not statistically significant (P>0.05), suggesting that i.n. administration of a single dose of inactivated H7N9 vaccine did not induce a significant cellular immune response, as least at the dosage used in this study. By contrast, the mice immunized with the H7N9 vaccine plus cGAMP developed a significant number of IFN-γ secreting splenocytes, with an average of seven times more than the vaccine alone groups. In conclusion, intranasal administrated of whole-virion H7N9 influenza vaccine with cGAMP as a mucosal adjuvant in mice could induce an increased systemic and mucosal antibody and T cell responses that confer better protection against a high lethal dose challenge of homologous influenza virus.

Intranasal Administration of cGAMP Increases Expression of Innate Immunity Cytokines of Mice in Lungs

To assess the ability of cGAMP in influencing expression of innate immunity cytokines, forty mice were randomized into 8 groups, with 5 mice in each group, mice were immunized as described above. Lung homogenates of mice in each group were collected at 24 h post-immunization for detection of the expression of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α. As shown in the **Figure 3**, intranasal immunization of inactivated H7N9 vaccine in a relatively high-dose alone could induce a certain level expression of IL-1β, IL-6, or TNF-α in mice lung homogenates (compared to the control group), while all the cGAMP adjuvanted groups induced a higher expression of innate immunity cytokines, as compared to the groups without cGAMP after 24 h ($P < 0.05$). The greater expression of IL-1β, IL-6 or TNF-α was directly associated to the presence of cGAMP. However, the expression of IL-1β, IL-6, or TNF-α did not cause obvious pathological and histological change in lung tissues of mice (**Figure S2**). This result suggests that the enhancement of mucosal and systemic immune responses to whole-virion H7N9 influenza vaccine by cGAMP was very likely due to the innate

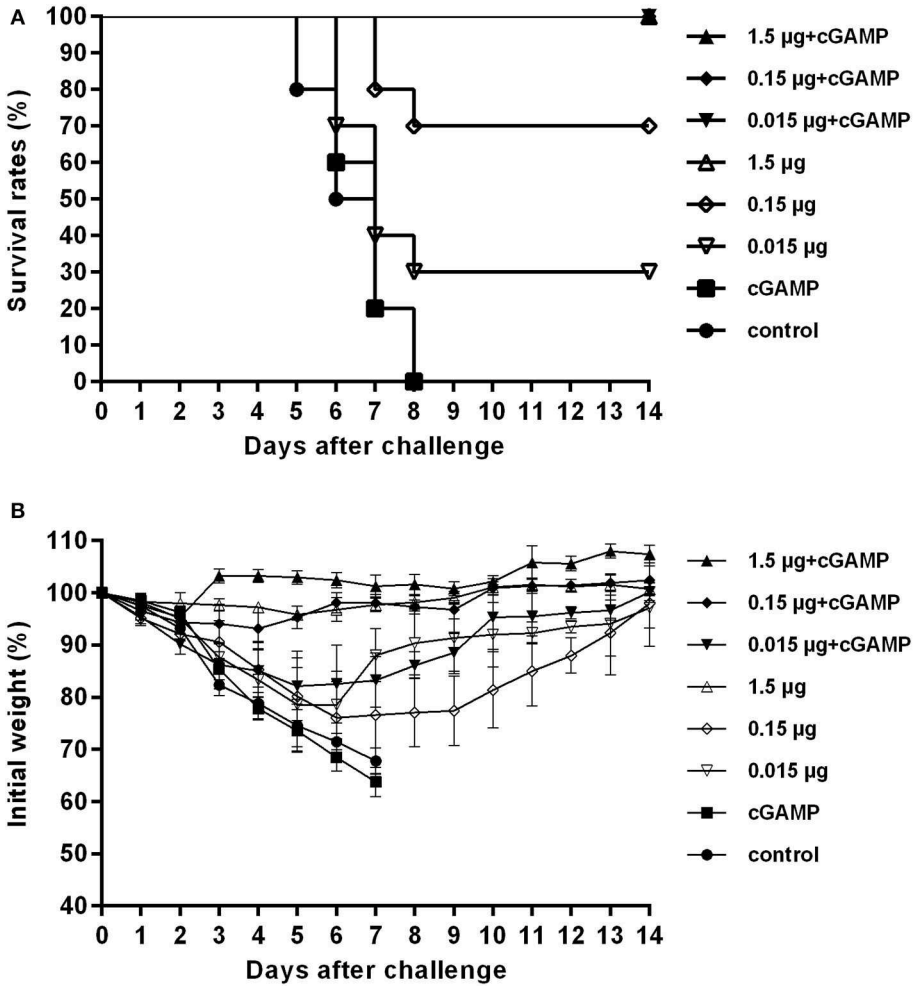

FIGURE 1 | cGAMP adjuvanted vaccine offers improved protection against a high lethal dose challenge of homologous virus. Mice were intranasally immunized once with various doses of whole-virion H7N9 influenza vaccine with or without cGAMP. Three weeks after immunization, mice were challenged intranasally with a high lethal dose (40 × LD_{50}) of mouse adapted Sh2/H7N9 influenza virus. Survival rates **(A)** and body weight changes **(B)** of mice were measured daily for 2 weeks after challenge.

immune recognition of cGAMP and the expression of innate immune mediators.

cGAMP Adjuvanted Vaccine Induces Improved Cross Protection Against a Lethal Dose Challenge of the Heterosubtypic Virus

Intranasal administration of a whole inactivated influenza virus vaccine was proved to be a promising way to induce a broad spectrum of heterosubtypic immunity against influenza A virus. To explore whether the addition of cGAMP could enhance the cross protection ability of the whole inactivated H7N9 influenza vaccine against a heterosubtypic influenza virus infection, a two-dose immunization regimen was used since a single dose of 1.5 μg vaccine plus cGAMP could not provide good protection against a heterosubtypic virus challenge (data not shown). Mice were intranasally immunized with either 1.5 ug HA of H7N9 vaccine alone or with 5 ug 2′-3′-cGAMP twice on day 0 and 21, and an unimmunized group was used as an negative control group. Three weeks after the last immunization, all the mice were i.n. challenged with 5×LD_{50} of mouse adapted A/Puerto Rico/8/34 (H1N1), A/Guizhou/54/1989(Gz54/H3N2) and A/Chichen/Jiangsu/11/ 2002 (H9N2) viral suspension (as shown in **Table 3**). Compared to the control group, a full protection against a lethal challenge of all the three virus strains was obtained in the adjuvanted vaccine immunized group, while the protection rates against H1N1, H3N2, and H9N2 were 60%, 50 and 60% respectively in the non-adjuvanted vaccine groups. Moreover, the lung virus titers were considerably reduced in mice immunized with the whole inactivated vaccine plus cGAMP, in comparison to mice that were vaccinated with the vaccine alone on day 3 post challenged with any of the heterosubtypic viruses. These collective results show that i.n administration of whole inactivated H7N9 vaccine in combination with cGAMP can significantly reduce the lung virus load, and confer

TABLE 2 | Serum and mucosal antibody responses in mice by intranasal administration of inactivated H7N9 vaccine with or without cGAMP adjuvant.

Group	Dose and adjuvant	Virus-specific antibody or HAI antibody titer			
		Virus-specific antibody titer (ELISA, 2^n)[a]			HAI antibody titer[a]
		Serum IgG	Nasal wash IgA	lung hemogenate IgA	Serum HAI
A	1.5μg + cGAMP	$8.60 \pm 0.56^{b,c}$	$5.40 \pm 0.55^{b,c}$	$3.60 \pm 0.55^{b,c}$	$448 \pm 175.27^{b,c}$
B	1.5μg	7.00 ± 0.71^{b}	2.60 ± 1.14^{b}	2.20 ± 0.45^{b}	64 ± 21.91^{b}
C	0.15μg + cGAMP	$6.60 \pm 1.14^{b,c}$	$4.00 \pm 1.00^{b,c}$	$2.20 \pm 0.84^{b,c}$	$48 \pm 17.89^{b,c}$
D	0.15μg	4.80 ± 0.84^{b}	1.80 ± 0.84^{b}	ND	20 ± 0.00^{b}
E	0.015μg +cGAMP	$4.20 \pm 0.84^{b,c}$	ND	ND	$16 \pm 5.48^{b,c}$
F	0.015μg	2.80 ± 0.84^{b}	ND	ND	ND
G	cGAMP	NT	NT	NT	NT
H	control	NT	NT	NT	NT

Mice were intranasally immunized once with various doses of whole-virion H7N9 influenza vaccine with or without cGAMP. Three weeks after the immunization, serum, nasal wash, and lung hemogenate specimens of 5 mice in each group were prepared. The titers of virus specific IgG and hemagglutination inhibition (HAI) antibodies in serum, virus specific IgA in nasal wash and lung hemogenate were detected by ELISA or HI assay. An ELISA Ab titer was expressed as the highest dilution giving a positive reaction. ND, not detected; NT, not tested.

[a] *Results are expressed as mean ± SD of five tested mice in each group.*

[b] *Displays significant difference compared with mouse in control groups ($P < 0.05$).*

[c] *Displays significant difference compared with mouse in the corresponding non-adjuvanted groups ($P < 0.05$).*

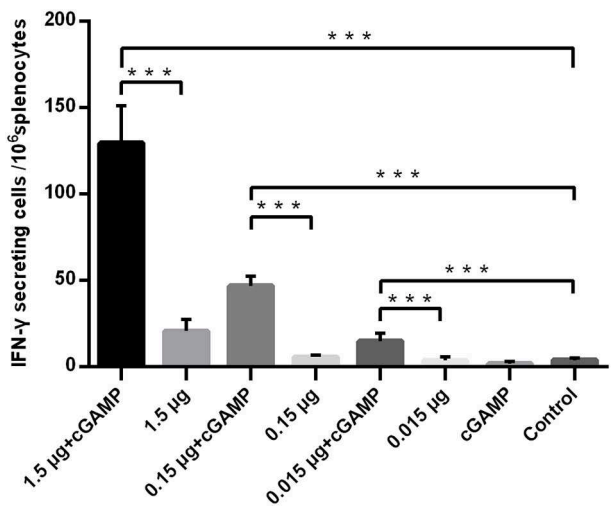

FIGURE 2 | cGAMP adjuvant enhances the T cell responses of whole-virion H7N9 influenza vaccine. Mice were intranasally immunized once with various doses of whole-virion H7N9 influenza vaccine with or without cGAMP. The amount of IFN-γ secreting splenocytes of immunized mice after stimulated with whole-virion H7N9 influenza vaccine was measured by ELISpot three weeks after immunization. Data shown as mean numbers of spot-forming cells (SFCs) ± SD. $n = 5$ per immunized group. Each sample was tested in triplicates. ***$P < 0.001$ (one-way ANOVA and Tukey's multiple comparison test).

broad spectrum protection against heterosubtypic influenza A viruses.

cGAMP Enhanced Cross-Reactive T Cell Response Against Virus Conserved Protein May Be Correlated With the Cross Protection

We examined whether it was possible to induce broad-spectrum cross-reactive HAI antibodies by intranasal administration of inactivated H7N9 in combination with cGAMP adjuvant. Mice were intranasally immunized as described above. The serum HAI antibody titers against the homo- and heterologous viruses were detected HI assay in 3 weeks after the last immunization. The results showed that a two-dose regimen of the H7N9 vaccine immunization could induce a higher level of HAI antibodies against homologous viruses, which was significantly higher than that induced by a single immunization with the same dose. In addition, the immuno-enhancing effect of cGAMP was also observed in the cGAMP adjuvanted vaccine group. However, neither the adjuvant nor the non-adjuvant group could induce detectable cross-reactive HAI antibodies against the H1N1, H3N2 and H9N2 virus (**Figure 4**). A further serum transfer study showed that transferring immune serum from cGAMP adjuvant vaccine immunized mice alone did not confer cross protection against a heterosubtypic virus to recipient mice (data not shown).

Since we did not detect cross-reactive HAI antibodies that could mediate cross protection in the immunized mice, the T cell responses against the conserved internal antigens nucleoprotein (NP) of the virus which has been proven to be able to mediate cross protection against heterosubtypic influenza virus was detected by IFN-γ ELISpot assay. The splenocytes of mice were isolated 3 weeks after the last immunization, and were, respectively, stimulated with whole inactivated influenza virus vaccine, a NP derived MHC-I epitope peptide and a pool of three NP derived MHC-II epitope peptides, which were corresponded to detection of virus specific T cell response, NP specific IFN-γ secreting $CD8^+$ T cell response and NP specific IFN-γ secreting $CD4^+$ T cell response. The results are shown in **Figure 5**. The considerable amounts of virus specific IFN-γ secreting T cells, NP specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells were induced by two doses of i.n immunization with the whole inactivated H7N9 vaccine. As expected, the cGAMP could effectively increase the number of NP specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells induced by the whole inactivated H7N9 vaccine.

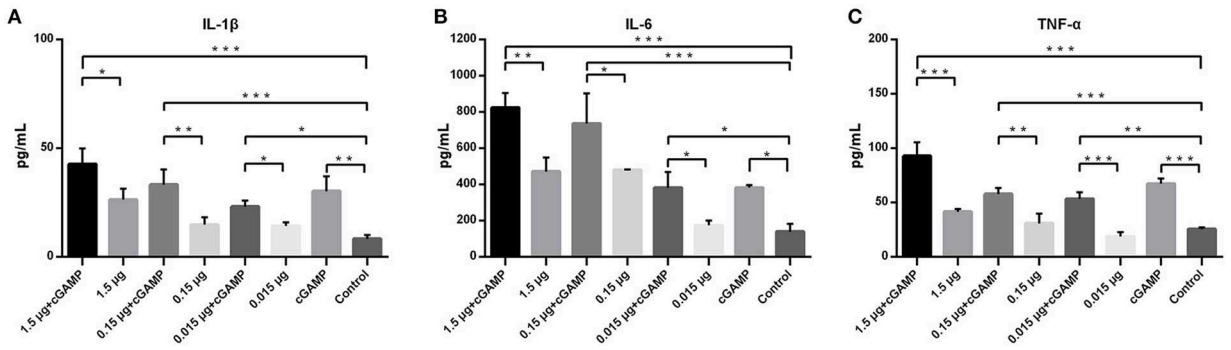

FIGURE 3 | cGAMP adjuvant enhances the expression of mouse lung innate immune cytokines. Mice were intranasally immunized once with various doses of whole-virion H7N9 influenza vaccine with or without cGAMP. The lung homogenates of immunized mice were collected for IL-1β, IL-6, and TNF-α measurement by ELISA 24 h after immunization. Data shown as mean ± SD. $n = 5$ per immunized group. Each sample was tested in triplicates. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ (one-way ANOVA and Tukey's multiple comparison test).

TABLE 3 | Protection against a lethal dose challenge of the heterosubtypic viruses in mice by intranasal administration of whole-virion H7N9 influenza vaccine combined with cGAMP.

Group	Dose and adjuvant	A/PuertoRico/8/1934 (H1N1)		A/Guizhou/54/1989(Gz54/H3N2)		A/Chicken/JiangSu/07/2002 (H9N2)	
		Lung virus titer[a] ($log_{10}TCID_{50}$/ml)	No. of survivors/no. tested	Lung virus titer[a] ($log_{10}TCID_{50}$/ml)	No. of survivors/no. tested	Lung virus titer[a] ($log_{10}TCID_{50}$/ml)	No. of survivors/no. tested
A	1.5μg + cGAMP	5.44 ± 0.30[b,c]	10/10[b,c]	6.14 ± 0.62[b,c]	10/10[b,c]	6.25± 0.38[b,c]	10/10[b,c]
B	1.5μg	9.19 ± 0.31[b]	6/10[b]	9.00 ± 0.46[b]	5/10[b]	7.00± 0.29[b]	6/10[b]
C	Control	10.13 ± 0.52	0/10	11.25 ± 0.35	1/10	8.05± 0.37	0/10

Mice were intranasally immunized with two doses of 1.5 ug HA of whole-virion H7N9 influenza vaccine with or without 5 ug 2′-3′-cGAMP on day 0 and 21. Three weeks after the last immunization, mice were challenged with a lethal dose (5×LD_{50}) of mouse adapted A/Puerto Rico/8/34 (H1N1), A/Guizhou/54/1989(Gz54/H3N2) and A/ Chichen/Jiangsu/11/ 2002 (H9N2) influenza virus. Lung hemogenate from 5 mice in each group were collected 3 days post-infection for titration of lung virus. The survival rates of mice 2 weeks post-infection were determined.

[a] *Results are expressed as mean ± SD of five tested mice in each group.*

[b] *Displays significant difference compared with mice in control groups ($P < 0.05$).*

[c] *Displays significant difference compared with mice in the corresponding non-adjuvanted groups ($P < 0.05$).*

We further elevated the expression of IFN-γ and granzyme B, which were used as the markers of activated cytotoxic T cells, in the lung homogenates of mice at 3 day post a lethal dose challenge with the heterosubtypic virus [A/PR/8/34 (H1N1)]. The results in **Figure 6** showed that the mice i.n. immunized with cGAMP adjuvanted vaccine had significantly higher levels of IFN-γ and granzyme B expression in response to the viral challenge than those in the control and the non-adjuvant group. This indicated that there were more activated cytotoxic T cells recruited to the infection sites (lung tissue and nasal mucosa), mediating viral clearance in early stage post infection. Based on the above results, we speculate that the enhanced cross-reactive NP, or other virus conserved protein specific $CD4^+$ and $CD8^+$ T cell response induced by i.n administration of inactivated H7N9 vaccine with GAMP adjuvant may be closely related to cross protection in the absence of cross-neutralizing antibodies.

cGAMP Adjuvanted Influenza Vaccine Provides Long-Term Cross Protection Against Heterosubtypic Influenza a Viruses Challenge

We investigated the long-term cross protection ability against heterosubtypic influenza A virus challenge after i.n. immunized of mice with inactivated H7N9 vaccine by using cGAMP as an adjuvant. Thirty mice were randomized into 3 groups, mice were immunized with two doses of 1.5 μg inactivated whole-virion H7N9 influenza vaccine, either with or without 5 ug 2′-3′-cGAMP as an adjuvant, and an unimmunized group was used as an negative control group. Six months after the last immunization, the mice were challenged with 5×LD_{50} of A/Puerto Rico/8/34 (H1N1) influenza virus, and survival rates were observed for 2 weeks. The results presented in **Figure 7** indicated that i.n. immunized mice with inactivated H7N9 vaccine plus cGAMP provided up to 80% protection against a heterosubtypic influenza vchallenge in mice, which was significantly higher than the non-adjuvant group with a protection rate of 30% ($P < 0.05$), while the control group failed to provide any protection and the body weight of mice continued to decline, resulting in all mice being dead within 8 days after the challenge. Moreover, the adjuvant group showed relatively mild weight loss and faster recovery following challenge compared to the groups without cGAMP. These results strongly suggest that immunization with a cGAMP adjuvanted influenza vaccine can provide more effective and longer-term protection against lethal challenges of heterosubtypic influenza virus.

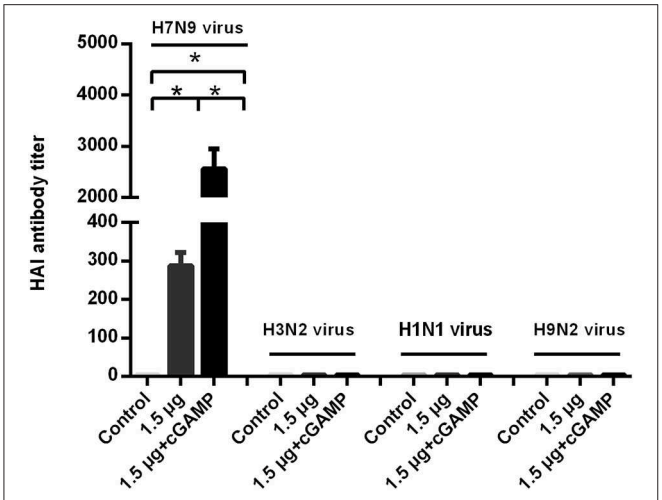

FIGURE 4 | cGAMP adjuvanted H7N9 influenza vaccine does not induce detectable cross-reactive hemagglutination inhibition antibodies against heterosubtypic virus. Mice were intranasally immunized with two doses of 1.5 ug HA of whole-virion H7N9 influenza vaccine with or without cGAMP on day 0 and 21. Three weeks after immunization, serum HI antibodies against homologous and heterosubtypic of H3N2, H1N1, H9N2 viruse were determined by HI assay. Data shown as mean titer ± SD. $n = 5$ per immunized group. Each sample was tested in triplicates. *$P < 0.05$ (one-way ANOVA and Tukey's multiple comparison test).

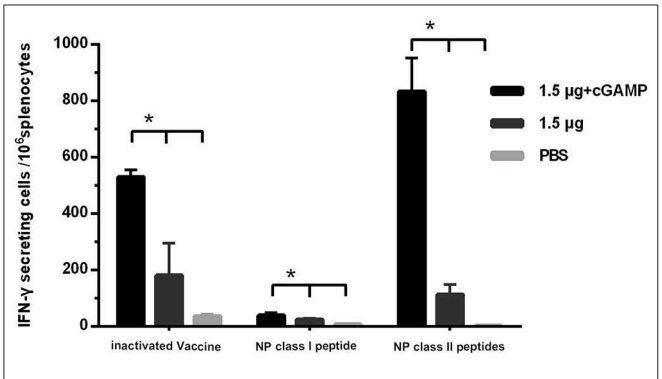

FIGURE 5 | cGAMP adjuvant enhances the T cell response against virus conserved protein. Mice were intranasally immunized with two doses of 1.5 ug HA of whole-virion H7N9 influenza vaccine with or without cGAMP on day 0 and 21. The amount of IFN-γ secreting splenocytes of immunized mice after stimulated with whole-virion H7N9 influenza vaccine, NP derived MHC-I epitope peptide or a pool of three NP derived MHC-II epitope peptides was measured by ELISpot three weeks after immunization. The splenocytes of immunized mice were isolated for IFN-γ measurement by ELISpot. Data shown as mean numbers of spot-forming cells (SFCs) ± SD. $n = 5$ per immunized group. Each sample was tested in triplicates. *$P < 0.05$ (one-way ANOVA and Tukey's multiple comparison test).

DISCUSSION

Currently, the epidemiological and risk assessments indicate a strong potential for the H7N9 virus to pose a public health risk (5). The development of effective vaccines is of great importance for the prevention and control of a possible H7N9 pandemic. Current influenza vaccines are usually non-adjuvanted, but the addition of an adjuvant may improve vaccine immunogenicity and permit dose-sparing, which may be critical for managing vaccine supplies during influenza pandemics (22). Previous studies have confirmed that the immunogenicity of H7N9 virus is relatively low in humans, and the usage of many different types of novel adjuvants may be necessary to improve the immunogenicity of H7N9 vaccines (9, 23, 24).

In this study, we used the novel STING agonist cGAMP as an adjuvant for the whole virus inactivated H7N9 vaccine. The results showed that cGAMP can effectively enhance the vaccine-induced serum-specific antibody and HAI antibody response in intranasally immunized mice. The antibody response induced by utilizing a cGAMP adjuvant achieved a dose-sparing effect of about 10 times higher than that of vaccine alone. That is, the serum antibody levels induced by 0.015 μg with cGAMP and 0.15 μg with cGAMP groups were comparable to the serum antibody levels induced by the 0.15 μg and 1.5 μg none adjuvanted vaccine groups, respectively. This finding may be of great value for the application of pre-pandemic influenza vaccines such as H7N9 vaccines (22). More importantly, we demonstrated that the level of mucosal antibodies induced by the vaccine supplemented with cGAMP adjuvant was significantly higher than that of the unadjuvanted group, which may play a decisive role in the first-time antiviral protection. In addition, the cGAMP was also shown to be effective in enhancing vaccine induced T cell immune responses, represented by higher induction of a strongly enhanced IFN-γ response in splenocytes from immunized mice.

The cGAMP acts as an agonist that activates the STING signaling pathway and the downstream NF-κB and IRF3 pathways to induce cytokine production to further promote adaptive immune responses via different molecular mechanisms (15, 18, 25). By examining the levels of innate immune-related cytokines in the lungs of mice at an early time post immunization, we found that intranasal administration of cGAMP can induce and increase the production of innate immune-related cytokines in lung tissues, which is consistent with previous reports by other groups using intramuscular, sublingual and skin immunization routes (16, 18). Given the fact that cGAMP acts as a natural small molecule, with its structure of phosphoric acid containing diester bonds that are easily degraded by phosphodiesterase *in vivo* (19, 26), the role of cGAMP is functional for only a short time without side effects. It is theorized that perhaps the moderate expression of these innate immune-related cytokines is important for vaccine-induced immune enhancement. Our results indicate that it is the enhanced humoral, cellular and mucosal immune responses induced by the cGAMP vaccine that protect mice during high lethal viral challenges.

The use of traditional inactivated influenza vaccines to induce broad-spectrum immune responses with novel immunization adjuvants or immunization strategies is one of the directions for development of broad-spectrum influenza vaccines. Several studies have demonstrated that intranasal immunization of inactivated influenza vaccine induces both humoral and cell-mediated immunity, suggesting that either or both of them might contribute to cross protection (11, 13, 27, 28). However, previous studies using mice intranasally vaccinated with inactivated influenza vaccines suggested that heterosubtypic immunity can occur in the absence of cross-neutralizing antibodies (29).

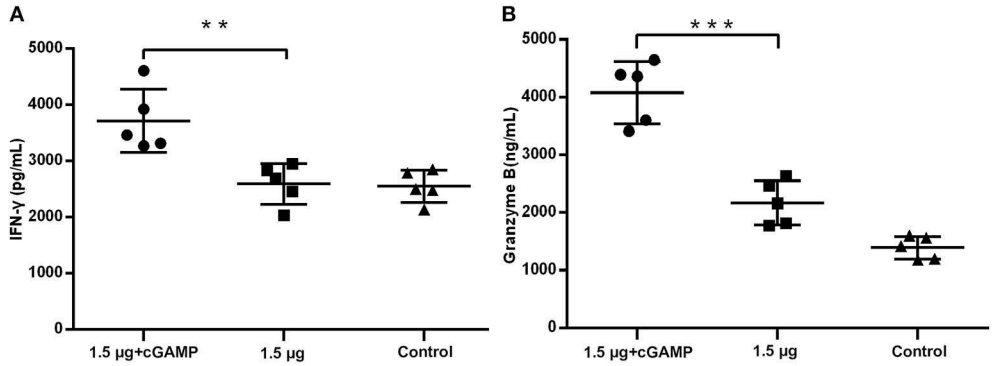

FIGURE 6 | cGAMP adjuvant upregulates production of cytotoxic T cell related cytokines in lung of mice post virus challenge. Mice were intranasally immunized with two doses of 1.5 ug HA of whole-virion H7N9 influenza vaccine with or without cGAMP on day 0 and 21. Three weeks after the last immunization, mice were challenged intranasally with a lethal dose (5 × LD50) of heterosubtypic A/PR/8/34 (H1N1) influenza virus. The concentration of IFN-γ **(A)** and granzyme B **(B)** in the whole lung homogenates of mice at three days post virus challenge were measured by ELISA. Data shown as mean ± SD. n = 5 per immunized group. Each sample was tested in triplicates. ***$P < 0.001$, **$P < 0.01$ (one-way ANOVA and Tukey's multiple comparison test).

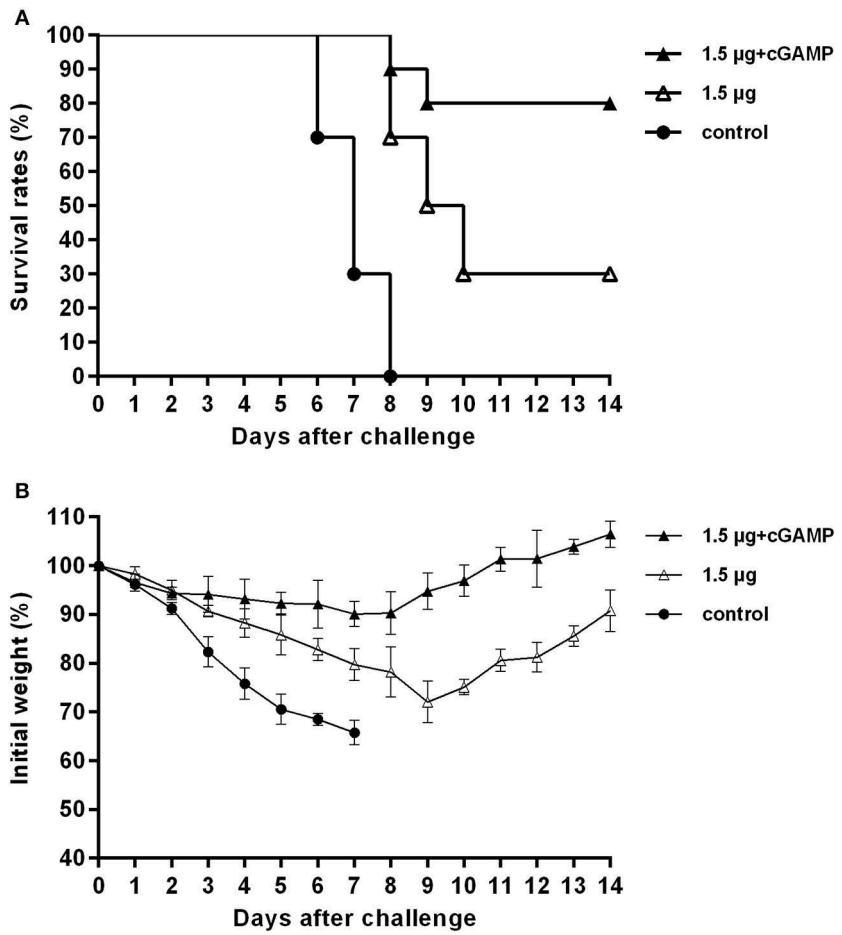

FIGURE 7 | cGAMP adjuvanted influenza vaccine provides long-term cross protection against heterosubtypic influenza A viruses challenge. Mice were intranasally immunized with two doses of 1.5 ug HA of whole-virion H7N9 influenza vaccine with or without cGAMP on day 0 and 21. Six months after the last immunization, mice were challenged intranasally with a lethal dose ($5 \times LD_{50}$) of heterosubtypic A/PR/8/34 (H1N1) influenza virus. Survival rates **(A)** and body weight changes **(B)** of mice after being challenged with a lethal dose of heterosubtypic influenza virus.

In the present study, we also found that mice immunized intranasally with two doses of cGAMP adjuvanted H7N9 influenza vaccine did not induce cross-reactive hemagglutination inhibition antibodies against H1N1, H3N2 and H9N2 influenza viruses, but could effectively protect mice against lethal challenge of these heterosubtypic influenza viruses.

Since our previous studies have shown that mice intranasally immunized with a recombinant internal conserved NP protein in combination with mucosal immune adjuvant (Cholera toxin B or C48/80) could produce better cross protection against a heterogeneous influenza virus challenge, and of which, the cellular immune response targeting these internal conservative antigens were regarded as the main factor mediating this cross protection against influenza (30, 31). In order to investigate the potential immune mechanism that mediate cross protection against heterosubtypic influenza virus in mice immunized with cGAMP adjuvanted H7N9 vaccine, we examined the specific T cell immune response against the NP, which was a highly conserved antigen with a high proportion in the whole virus inactivated vaccine. We found that intranasal co-administration of H7N9 vaccine with cGAMP could induce high levels of NP-specific IFN-γ-producing T cells, especially large amounts of IFN-γ-producing $CD4^+$ T cells, suggesting a considerable Th1 response. Previous studies by Ivana et al. using OVA antigen supplemented with cGAMP also suggested that cGAMP is an adjuvant that induces a Th1 biased response (16). In addition, recent studies have shown that cGAMP acts as a potent adjuvants *in vitro* and *in vivo*, enhancing the induction of functional antigen-specific $CD8^+$ T cell responses in mice and human cells (32). By further detecting the expression of IFN-γ and granzyme B in the lung tissue of immunized mice in an early stage (after receiving a heterologous influenza virus challenge), it was found that the cGAMP adjuvant group had more cytotoxic effector molecules in mice lung tissue in short-term after virus infection. These observations demonstrate that the cross-reactive NP-specific $CD8^+$ T and $CD4^+$ T cells can rapidly proliferate, differentiate, and be recruited to infection sites after viral infection, kill viral infected cells through direct killing, FasL, dependent, IFN-γ dependent or TRAIL dependent pathway and mediate clearance of virus (33–36).

In the present study, we showed that intranasal delivery of an inactivated H7N9 vaccine by using cGAMP as a mucosal adjuvant induces both systemic and mucosal immunity with an antigen dose-sparing effect. In addition, the cGAMP adjuvanted vaccine elicited high HAI antibody responses and effective protection against homologous viral challenge. Furthermore, the administration of cGAMP-vaccine induced a enhanced cross-reactive T cell responses that conferred cross protection against heterosubtypic influenza A virus challenges. Our results collectively suggest that the cGAMP may be a promising adjuvant for vaccines targeted against pandemic influenza and a mucosal vaccine for broad protection against divergent influenza A virus.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The protocol for the animal study (Protocol Number: 17-1250) was approved by the laboratory animal management committee, and the laboratory animal ethics and welfare protection group of Shanghai Institute of Biological Products. All animal procedures were carried out in accordance with the animal ethics guidelines of the Chinese National Health and Medical Research Council (NHMRC).

AUTHOR CONTRIBUTIONS

JL performed the majority of the experiments, evaluated the data, prepared the manuscript. XL and FX participated in lung virus titration. FG, YY, and MZ participated in immune response assay. ZC and WT conceived and designed the experiments, 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.2019.02274/full#supplementary-material

Figure S1 | SDS-PAGE analysis of inactivated whole-virion h7n9 influenza vaccine. The bulk of inactivated whole-virion H7N9 influenza vaccine with a total protein contentation of 400 ug/ml was treated with PNGase F, the optimal ratio of PNGase F to bulk was 1:50(v/v). After treatment, the vaccine sample was fractionated by SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue.

Figure S2 | Histologic analysis of mouse lung tissues after intranasal administration with cGAMP as adjuvant. Mice were intranasally administered once with with 5 μg **(A)**, 25μg **(B)**, 50 μg **(C)**, 100 μg **(D)** cGAMP or 1.5 μg inactivated whole-virion H7N9 influenza vaccine with or without 5 μg cGAMP **(E,F)**, the PBS **(G)** and LPS **(H)** groups were used as negative control and lung injury control, respectively. The lung tissues were removed for hematoxylin and eosin (HE) staining 24 h after administration. Representative photos are shown with 3 mice in each groups. Scale bar denotes 100 μm.

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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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Mincle and STING-Stimulating Adjuvants Elicit Robust Cellular Immunity and Drive Long-Lasting Memory Responses in a Foot-and-Mouth Disease Vaccine

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Conventional foot-and-mouth disease (FMD) vaccines exhibit several limitations, such as the slow induction of antibodies, short-term persistence of antibody titers, as well as low vaccine efficacy and safety, in pigs. Despite the importance of cellular immune response in host defense at the early stages of foot-and-mouth disease virus (FMDV) infection, most FMD vaccines focus on humoral immune response. Antibody response alone is insufficient to provide full protection against FMDV infection; cellular immunity is also required. Therefore, it is necessary to design a strategy for developing a novel FMD vaccine that induces a more potent, cellular immune response and a long-lasting humoral immune response that is also safe. Previously, we demonstrated the potential of various pattern recognition receptor (PRR) ligands and cytokines as adjuvants for the FMD vaccine. Based on these results, we investigated PRR ligands and cytokines adjuvant-mediated memory response in mice. Additionally, we also investigated cellular immune response in peripheral blood mononuclear cells (PBMCs) isolated from cattle and pigs. We further evaluated target-specific adjuvants, including Mincle, STING, TLR-7/8, and Dectin-1/2 ligand, for their role in generating ligand-mediated and long-lasting memory responses in cattle and pigs. The combination of Mincle and STING-stimulating ligands, such as trehalose-6, 6′dibehenate (TDB), and bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), induced high levels of antigen-specific and virus-neutralizing antibody titers at the early stages of vaccination and maintained a long-lasting immune memory response in pigs. These findings are expected to provide important clues for the development of a robust FMD vaccine that stimulates both cellular and humoral immune responses, which would elicit a long-lasting, effective immune response, and address the limitations seen in the current FMD vaccine.

Keywords: PRR ligands, cytokines, adjuvants, foot-and-mouth disease, vaccine

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious viral disease that mainly affects cloven-hoofed livestock. This disease causes serious economic losses to the livestock industry, due to a rapid spread and high livestock mortality, resulting in low livestock productivity (1). Over 70 species of wildlife, including livestock ruminants such as cows, pigs, buffalos, camels, sheep, and goats, are susceptible to this disease. FMD is associated with high fever and causes blisters on the mouth, tongue, snout, nose, nipple, hoof, and other hairless areas of the skin (2).

Immunization with inactivated vaccines, which are used as a means of controlling the disease in countries afflicted by FMD, constitutes an important part of the contingency plans drawn up to meet emergency situations in FMD-free countries (3). Similar to other vaccines that were generated against viral diseases, several trials designed to generate a live attenuated vaccine for FMDV have failed due to unstable phenotypes, variable pathogenic profiles, risk of virus transmission, and failure to induce adequate protection (4). Inactivated vaccines are used against FMD globally. In order to produce an effective vaccine, an antigen purification process, which removes cellular contaminants as well as non-structural viral proteins (NSPs), is required to facilitate diagnostic testing that differentiates infected animals from vaccinated animals (DIVA). Since vaccine antigens consisting of dead viruses do not replicate or induce antibodies against NSPs, anti-NSP antibodies have often been used as markers of infection. The efficacy of inactivated vaccines was improved by including oil adjuvants (double or single oil emulsions). However, these vaccines exhibited certain limitations, such as the slow induction of antibodies to levels allowing for defense, low antibody titers, short-term persistence of antibodies, and low immunogenicity in pigs.

FMD vaccines focus on inducing humoral immune responses rather than cellular immune responses. But their protective effect is not perfect. While the period taken for induction of the humoral immune response, via the major neutralizing antibody, IgG, by FMD vaccines is 4–7 days (5), T cell-mediated cellular immune response is generated by innate immunity, where injection of Ag or infection with a pathogen activates innate immune cells within a few hours to 2–3 days (6). These cells then trigger inflammatory responses by secreting proinflammatory cytokines, chemokines, and costimulatory molecules. This innate, cell-mediated, immune response is amplified within 3 days and peaks after 3–7 days. It is an effective defense system that can recognize and clear the virus rapidly in the early stages of FMDV infection or reinfection. Moreover, current FMD vaccines have a short duration of antibody persistence following inoculation, requiring periodic vaccinations at intervals of 4–6 months. When intramuscularly administered to pigs in particular, these vaccines often cause lesions, such as fibrosis and granuloma in the inoculated muscles, indicating issues such as local side effects and low safety. Although studies related to FMD-related vaccines have focused on investigating the efficacy of vaccines in cattle, rather than in pigs, the immunogenicity induced by vaccination is lower in pigs than in cattle (7). Therefore, to overcome the limitations of the current commercial vaccines, the ideal vaccine design should have the following characteristics: simultaneous induction of both cellular and humoral immune responses, maintenance of high antibody titers through the induction of memory response, achievement of safety to reduce local side effects, and a new strategy for the development of adjuvants optimized for different livestock species.

Various adjuvant-related studies have investigated methods for improving protection against FMD, including an evaluation of the efficacy of FMD vaccines in pigs and goats using pattern recognition receptors (PRR) ligands such as Resiquimod (R848), poly(I:C) (8), muramyl dipeptide (MDP), monophosphoryl lipid (MPL), and β-glucan (9). Use of immune-boosting agents such as rapeseed oil and ginseng root saponin (10) as well as commercially available adjuvants such as ISA 201, ISA 206, Emulsigen-D, and Carbigen have also been evaluated. However, thus far, adjuvant-induced perfect immunity has not been found. In an effort to increase immunogenicity, focus was placed on the induction of cellular and humoral immune responses, as well as human vaccines rather than FMD vaccines, and the following were studied and utilized as adjuvants (11, 12): (1) vaccine delivery systems such as oil emulsions, surfactants, liposomes, virosomes, and immune-stimulating complexes; (2) immune-boosting agents such as saponin, aluminum hydroxide ($Al(OH)_3$), and potassium phosphate; (3) receptor-specific immune stimulators such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and ligands for C-type lectin receptors (CLRs); and (4) a variety of cytokines such as IL-1, IL-2, IL-6, IL-18, TNFα, IFNγ, and GM-CSF. Some of these are currently in use or undergoing clinical trials for use as vaccine adjuvants for the prevention and treatment of various human diseases such as cancer, tuberculosis, hepatitis B, malaria, influenza, human immunodeficiency virus, and the herpes simplex virus (13, 14), but none have been utilized as a component of FMD vaccines. Moreover, since different adjuvants have different modes of action, it is important to understand the immunological mechanism underlying the role of these adjuvants in order to facilitate the development of FMD vaccines using a new strategy that may induce strong cellular and humoral immune responses simultaneously.

Our group conducted intensive studies to investigate different serotypes of FMDV Ag-mediated cellular immune response *in vivo* and *in vitro* (murine, bovine, and porcine immune cells) as well as the effectiveness of various PRR ligands and cytokines as adjuvants in mice. We also examined their ability to induce cellular and humoral immune responses in mice and analyzed related mechanisms to elucidate the differences in immune responses among livestock species, such as cattle and pigs. Therefore, in order to develop specific adjuvants optimized for each livestock species and produce novel FMD vaccines that included these adjuvants, this study pursued the following objectives: evaluate memory response induction by adjuvants, including PRR ligands and cytokines; screen adjuvants that stimulate immune responses in peripheral blood mononuclear cells (PBMCs) isolated from the whole blood of cattle and pig; evaluate the composition of the experimental vaccines, including

adjuvants selected for their ability to induce a humoral immune response *in vivo* (cattle and pigs); propose a new strategy for the development of FMD vaccines.

MATERIALS AND METHODS

Antigen (Ag) Purification and Inactivation

Ags were prepared by cultivating the FMD virus (FMDV) O/TWN/97-R (GenBank AY593823 for P1) in BHK-21 cells according to the method described by Lee et al., with modifications (15). To initiate viral infection, the culture medium was replaced with serum-free Dulbecco's modified Eagle's medium (DMEM; Cellgro, Manassas, VA, USA), and the cells were inoculated with the virus and incubated for 1 h at 37°C in a 5% CO_2 atmosphere. All extracellular viruses were then removed. At 24 h post-infection, the viruses were inactivated with two treatments of 0.003 N binary ethylenimine for 24 h in a shaking incubator (16) and concentrated using polyethylene glycol (PEG) 6000 (Sigma-Aldrich, St. Louis, MO, USA). The virus concentrate was layered onto 15–45% sucrose density gradients and centrifuged (17). After ultracentrifugation, the bottoms of the centrifuge tubes were punctured and 1 ml fractions were collected. The presence of FMDV particles in a sample of each fraction was confirmed by optical density using a lateral flow device (BioSign FMDV Ag; Princeton BioMeditech, Princeton, NJ, USA). Prior to its use in the experiment, the pre-PEG treatment supernatant was passed through ZZ-R and BHK-21 cells at least twice to ensure that no cytopathic effect (CPE) occurred, thereby confirming the absence of any live viruses in the supernatant.

PRR Ligands and Cytokines

PRR ligands were purchased from InvivoGen (InvivoGen, San Diego, CA, USA), and cytokines were purchased from Mitenyi Biotec (Miltenyi Biotec, Bergisch Gladbach, Germany) and R& D Systems (R&D Systems, Minneapolis, MN, USA). ISA 206, an oil emulsion, was purchased from Seppic Inc. (Paris, France), and aluminum hydroxide gel (Alhydrogel®) and Quil-A were purchased from InvivoGen.

Mice

Age- and sex-matched wild-type C57BL/6 mice (7-week-old females) were purchased from KOSA BIO Inc. (Gyeonggi, Korea). All mice were housed in microisolator cages in a specific pathogen-free animal facility at biosafety level 3 (ABSL3) at the Animal and Plant Quarantine Agency. The studies were performed according to institutional guidelines and with approval from the Ethics Committee of the Animal and Plant Quarantine Agency.

Memory Immune Response Mediated by PRR Ligands and Cytokines in Mice

To evaluate the potential of PRR ligands and commercially available recombinant cytokines as vaccine adjuvants, and to investigate their protective effect against FMDV infection and their ability to induce a memory response, experiments were performed using the strategy shown (**Figure 1A**) ($n = 11$ per group). O/TWN/97-R Ag was used as inactivated FMDV Ag. The vaccine composition for the positive control (PC) group was as follows: O/TWN/97-R Ag (15 μg/dose/ml, 1/160 dose), ISA 206 (50%, w/w), 10% $Al(OH)_3$, and 15 μg/mouse of Quil-A for a total volume of 100 μl. All experimental group mice received vaccines with the same composition as the PC group, with the addition of either PRR ligands or recombinant cytokines as an adjuvant. Mice in the negative control group received an equal volume of phosphate-buffered saline (PBS, pH 7.0) administered via the same route. Briefly, the mice were vaccinated intramuscularly in the thigh muscle. Later, 56 days post vaccination (dpv), the mice were challenged with FMDV (100 LD_{50} of O VET 2013, ME-SA topotype) by intraperitoneal (I.P.) injection. Their survival rates and body weights were monitored up to 7 dpc. In addition, serum and peritoneal exudate cells (PEC) sampled from mice at 0, 28, and 56 dpv were analyzed via structural protein enzyme-linked immunosorbent assay (SP ELISA), virus neutralization (VN) titers, and PEC subpopulations to determine the ability to induce cellular and humoral immune responses. In order to identify whether FMDV O Ag-specific T cell responses and memory T cell responses were amplified by Ag re-stimulation, we isolated pre (0 dpi) and post Ag injection (28 dpi) mice PEC (PC group). T cells were purified from isolated PEC (Pan T Cell Isolation Kit II, Miltenyi Biotec) and sorted via flow cytometry (purity > 98%). T cells were cultured at 37°C and 5% CO_2 in complete RPMI media (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (HyClone, Logan, Utah, USA), 10 mM HEPES (Gibco), 10 U/ml penicillin/streptomycin (Sigma-Aldrich), and 50 mM 2-mercaptoethanol (Sigma-Aldrich). Cells were subsequently treated *in vitro* with or without Ag (1 μg/ml) for 6 h. The percentage of $IFN\gamma^{pos}CD4^{+}$ T cells and $IFN\gamma^{pos}CD8^{+}$ T cells was compared via flow cytometry as described in 2.5. ELISA for IFNγ (R&D Systems, Minneapolis, MN, USA) was also performed on T cell culture supernatants according to the manufacturer's instructions.

Flow Cytometric Analysis

In order to analyze PEC subpopulations, single-cell PEC suspensions ($0.5–1 \times 10^6$ cells in PBS supplemented with FBS) were incubated with purified anti-CD16/32 antibodies (Abs) (FcγRII/III block, Clone. 2.4G2; eBioscience, San Diego, CA, USA) to block non-specific staining. PEC was immunostained with fluorochrome-conjugated Abs to CD3 (Miltenyi Biotec, Clone. REA641), CD4 (Miltenyi Biotec, Clone. REA604), CD8a (Miltenyi Biotec, Clone. 53-6.7), CD44 (Miltenyi Biotec, Clone. REA664), CD62L (Miltenyi Biotec, Clone REA828), CD27 (Miltenyi Biotec, Clone. REA499), anti- γδ TCR (Miltenyi Biotec, Clone REA633), CD335 (NKp46) (Miltenyi Biotec, Clone. REA815), CD11c (Miltenyi Biotec, Clone REA754), anti-MHC Class II (Miltenyi Biotec, Clone. REA813), CD11b (Miltenyi Biotec, Clone REA592), and anti-F4/80 (Miltenyi Biotec, Clone. REA126). For intracellular staining of cytokines, cells were stimulated by PMA and ionomycin in the presence of Golgi-stop (BD Bioscience, Franklin Lakes, NJ, USA) in complete RPMI medium for 4 h. After stimulation, cells were washed, and surface molecules were stained. Cells were then fixed with Intracellular (IC) Fixation Buffer (eBioscience), washed with Perm Buffer,

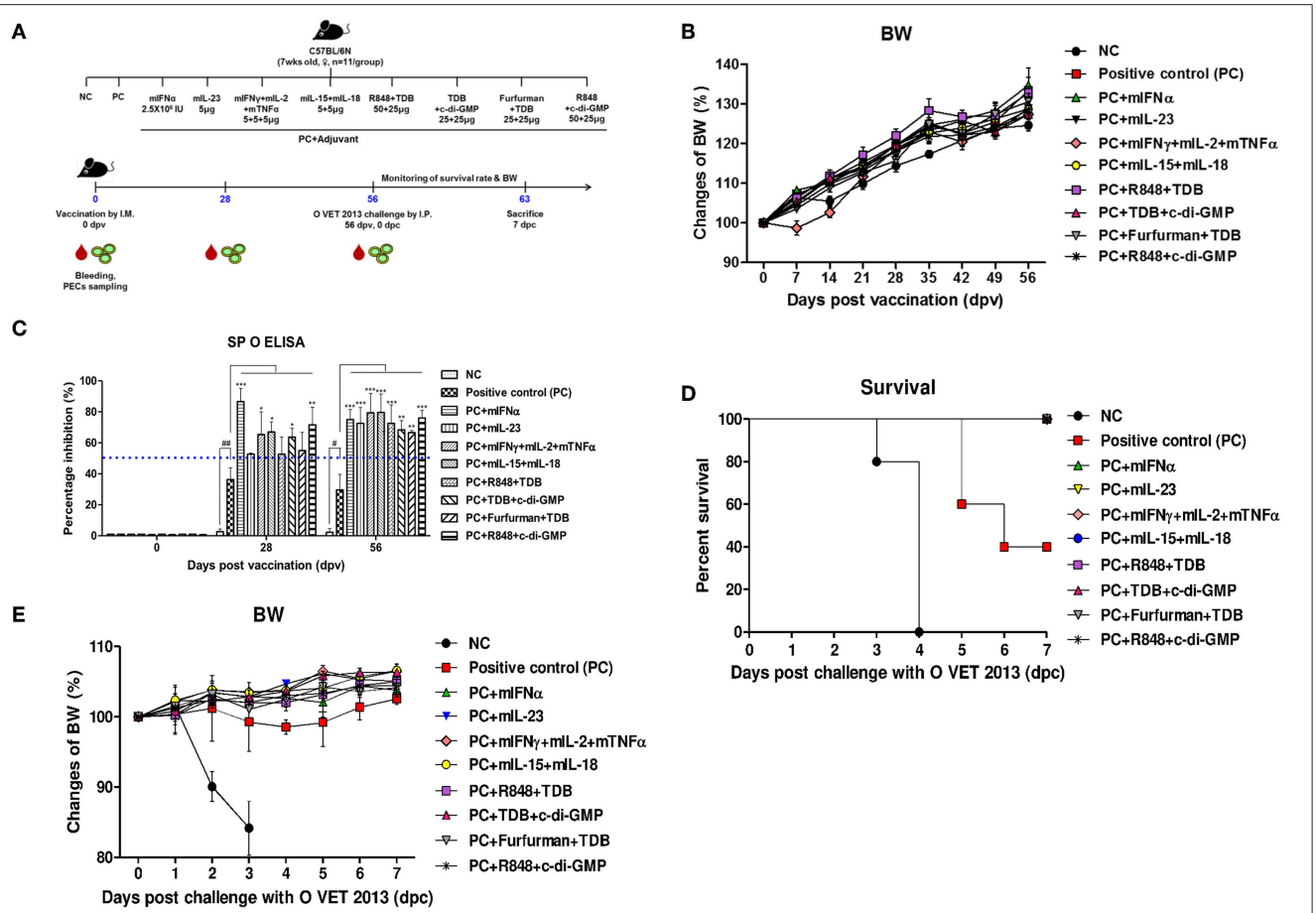

FIGURE 1 | Adjuvanticity of PRR ligands and pro-inflammatory cytokines; significant enhancement of the memory response in mice against FMDV infection C57BL/6 mice were administered either a combination of PRR ligands or cytokines with the vaccine based on the vaccine composition of the positive control group. The PRR ligands and cytokines used in the experiment were as follows: TDB (Mincle agonist), c-di-GMP (STING agonist), Furfurman (Dectin-2 agonist), R848 (TLR-7/8 agonist), mIFNα, mIL-23, mIFNγ, mIL-2, mTNFα, mIL-15, and mIL-18. A negative control group of mice was injected with the same volume of PBS as the vaccine, and a positive control group received 11.7 ng (1/160 of the dose for cattle or pig use) of O/TWN/97-R Ag, ISA 206 (50%, w/w), 10% $Al(OH)_3$, and 15 μg Quil-A without PRR ligands and cytokines. The test vaccines were injected intramuscularly into mice that were later challenged with FMDV (100 LD_{50} O VET 2013) at 56 dpv. Blood sampling was performed on 28 dpv and 56 dpv for the serological assays. The survival rates and body weights were monitored for 7 dpc. **(A–E)** represent **(A)** the strategy for this study; **(B)** antibody titer by SP O ELISA; **(C)** changes in body weight post vaccination; **(D)** survival rate against FMDV (O VET 2013); **(E)** changes in body weight post challenge with O VET 2013. The data are the mean ± SEM of triplicate measurements; statistical analyses were performed using two-way ANOVA with Bonferroni correction; $^{\#}$, $^{*}p < 0.05$, $^{\#\#}$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

and stained with anti-IFNγ (Miltenyi Biotec, Clone. REA638). Data were acquired via flow cytometry (MACSQuant® Analyzer 10, Miltenyi Biotec) and analyzed by FlowJo software vX 0.7 (TreeStar, Ashland, OR, USA). Cell counts were performed in duplicate following the addition of Trypan blue dye using a Vi-CELL Series Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA).

PBMC Isolation

Bovine and porcine whole blood was donated by the Gyeonggi Veterinary Service Laboratory. FMD antibody-seronegative animals were used as donors ($n = 4$/group for bovine PBMC; $n = 6$/group for porcine PBMC). Whole blood (15 ml/each donor) was independently collected in a BD Vacutainer heparin tube (BD, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and PBMCs were isolated using Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) gradient centrifugation. Residual red blood cells were lysed by treating them with ACK (ammonium-chloride-potassium) lysing buffer (Gibco, Carlsbad, CA, USA). The PBMCs were suspended in Dulbecco's PBS without Ca^{2+} and Mg^{2+} (Gibco, Carlsbad, CA, USA), supplemented with 2% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), and counted using a volumetric flow cytometer (Miltenyi Biotec). All cells were freshly isolated directly before use, and no cryopreserved cells were used in any experiment. Purified PBMCs were then resuspended in RPMI-1640 (Gibco, Carlsbad, CA, USA) medium supplemented with 10% FBS (HyClone, Logan, Utah, USA), 3 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and 100 U/ml penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), plated at 1

$\times 10^4$ cells per well in 96-well plates, and incubated at 37°C with 5% CO_2. Following a 3 h incubation, the culture medium was replaced with a serum-free medium prior to stimulation with various PRR ligands and cytokines.

PRR Ligand and Cytokine Treatment

Bovine ($n = 4$) and porcine ($n = 6$) PBMCs were treated with PRR ligands and cytokines, as shown (**Supplementary Table 1**). After 96 h, the cell culture medium (supernatant) was harvested, and cytotoxicity [via lactate dehydrogenase (LDH) release] and cell proliferation [via 5-bromo-2′-deoxyuridine (BrdU) incorporation] were assessed.

PRR Ligand- and Cytokine-Mediated LDH Release Assay in Bovine and Porcine PBMCs

Cytotoxicity levels were detected in the supernatant of bovine and porcine PBMCs treated with PRR ligands and cytokines, as described above. An LDH release assay was performed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), following the manufacturer's protocol. The percentage of LDH release was calculated as follows: percentage of LDH release =100 × (absorbance reading of treated well—absorbance reading of untreated control)/(absorbance reading of maximal LDH release control—absorbance reading of untreated control). The lysis buffer provided by the kit was used to achieve complete cell lysis, and the supernatant from the lysis buffer-treated cells was used to determine maximum LDH release control.

BrdU Incorporation Assay in Bovine and Porcine PBMCs

The effects of PRR ligands and cytokines on the proliferation of bovine and porcine PBMCs were assessed using a BrdU Cell Proliferation Assay Kit (Cell Signaling Technology, Beverly, MA, USA) based on the incorporation of BrdU during DNA synthesis. Briefly, 10 μM BrdU was added to the cell culture and incubated for 4 h at 37°C. The cells were then fixed and incubated with an anti-BrdU mouse monoclonal antibody, followed by horseradish peroxidase-conjugated goat anti-mouse antibodies. The chromogenic substrate tetramethylbenzidine was used for color development. Absorbance was measured at a dual wavelength of 450/550 nm.

Cattle and Pigs

In order to evaluate the potential of PRR ligands and recombinant cytokines as vaccine adjuvants and to investigate their ability to induce cellular and humoral immune responses and long-term immunity, field experiments using cattle and pigs were conducted. For the field experiment, FMD antibody-seronegative animals from 2 farmhouses were used (the cattle were 5 months old and the pigs were 10 weeks old). The cattle and pigs were divided into 3 and 4 groups, respectively ($n = 5$/group). The animals were kept in closed containments during the study. The studies were performed according to institutional guidelines, with approval from the Ethics Committee of the Animal and Plant Quarantine Agency.

Immunization and Sampling

O/TWN/97-R Ag was used as the FMD Ag, and the vaccine composition for the positive control group was as follows: 1 ml vaccine prepared as a single dose, which included 15 μg of O/TNW/97-R Ag, ISA 206 (50%, w/w), 10% $Al(OH)_3$, and 150 μg Quil-A.

Vaccination was performed twice at a 28 days interval, and 1 ml of vaccine (1 dose) was administered via the deep intramuscular route on the necks of the animals. Blood samples were collected at 0, 14, 28, 56, 84, 112, 140, and 168 dpv from cattle and at 0, 14, 28, 42, 56, 70, and 84 dpv from pigs. The animals were monitored daily for body temperature, symptoms at vaccination site, and appetite. Serum samples were stored at −80°C until tests were performed.

Serological Assays

ELISA for the Detection of Structural Protein (SP) Antibodies

To detect SP antibodies in the sera, PrioCHECK FMDV type O (Prionics AG, Switzerland) was used. Absorbance in the ELISA plate was converted to a percent inhibition (PI) value. When the PI value was 50% or above, the animals were considered antibody positive.

Virus Neutralization Test

A virus neutralization test was performed according to the World Organization for Animal Health (OIE) manual (18). The sera were heat inactivated at 56°C for 30 min in a water bath. Cell density was adjusted to form a 70% monolayer, and 2-fold serial dilutions of sera samples (1:4–1:512) were prepared. The diluted sera samples were then incubated with a 100-tissue culture infectious dose $(TCID)_{50}$/0.5 ml homolog virus for 1 h at 37°C. After 1 h, a LF-BK (bovine kidney) cell suspension was added to all wells. After 2–3 days, CPE was checked to determine the titers, which were calculated as Log_{10} of the reciprocal antibody dilution required to neutralize 100 $TCID_{50}$ of the virus (19, 20).

Statistics

All quantitative data are expressed as mean ± SEM, unless otherwise stated. Between groups, statistical significances were assessed using two-way ANOVA followed by the Bonferroni *post-hoc* test and one-way ANOVA followed by Tukey's *post-hoc* test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Survival curves were built using the Kaplan-Meier method and differences were analyzed using the log-rank sum test. GraphPad Prism 5 (GraphPad, San Diego, CA, USA) software was used for all statistical analyses.

RESULTS

Inclusion of PRR Ligands and Cytokines as FMD Vaccine Adjuvants Induce Potent Memory Responses and Elicit a Protective Effect Against FMDV Infection in Mice

Mouse experiments were performed to evaluate the potential of PRR ligands and recombinant cytokines as FMD vaccine adjuvants and the induction of adjuvant-mediated memory immune response (**Figure 1A**). As indicated by SP O ELISA,

the group administered with PRR ligand and FMDV O Ag showed high antibody titers at 28 dpv (**Figure 1B**), and antibody titers were also significantly elevated in the experimental groups treated with rmIFNα ($p < 0.001$), rmIFNγ+rmIL-2+rmTNFα ($p < 0.05$), rmIL-15+rmIL-18 ($p < 0.05$), TDB+c-di-GMP ($p < 0.05$), and R848+c-di-GMP ($p < 0.01$) compared to the positive control group. Furthermore, at 58 dpv, significantly higher antibody titers were observed in all experimental groups vaccinated with cytokines and PRR ligands as adjuvants ($p < 0.01$ and $p < 0.001$, respectively). To determine the effect of the vaccination itself on body weight, mouse body weight was monitored once a week for 8 weeks (56 d) following vaccination, but no significant differences were found between the groups (**Figure 1C**).

Later, 56 days following vaccination, the mice were challenged with O VET 2013 and monitored for survival rates (**Figure 1D**) and changes in body weight (**Figure 1E**). Mice receiving PRR ligands and cytokines as vaccine adjuvants had a 100% survival rate without weight loss. By contrast, 100% of the mice in the negative control group died by 4 dpc, and the mice in the positive control group had a survival rate of 40%.

PRR Ligands and Cytokines Promote the Expansion of Memory Immune Cells

To investigate cellular and humoral immune responses mediated by PRR ligands and cytokines, the expansion of immune cells was analyzed using flow cytometry. $CD4^+$ T cells were expanded in the group supplemented with R848+TDB and TDB+c-di-GMP on 28 dpv and mIFN, mIL-15+mIL-18, R848+TDB, and TDB+c-di-GMP on 56 dpv (**Figure 2A**, **Supplementary Figure 3A**). $CD8^+$ T cells in the mIFNα-treated group had a lower absolute cell number than $CD4^+$ T cells but showed significantly higher cell expansion on 28 dpv (**Figure 2B**, **Supplementary Figure 3B**). The expansion of $CD44^{high}$ $CD62^{low}$ T cells, a memory T cell marker, increased rapidly at 56 dpv compared to 28 dpv. Although the cell number of these effector memory T cells increased in all the adjuvant treated groups at 56 dpv (**Figure 2C**, **Supplementary Figure 3C**), PRR ligands induced the expansion of these cells more significantly than cytokines at 28 dpv.

$CD44^{high}$ $CD27^{low}$ γδ T cells, known as memory γδ T cells, were expanded 8–12% by the addition of PRR ligands and cytokines, and no difference was observed between 28 and 56 dpv (**Figure 2D**, **Supplementary Figure 3D**). The expansion of $CD44^+CD27^+$ B cells, known as memory B cells, was significantly increased at 28 dpv in the mIL-23, R848+TDB, and TDB+c-di-GMP adjuvant groups. Overall, the absolute cell number was higher at 56 dpv compared to 28 dpv, and memory B cells were significantly expanded in all PRR ligand- and cytokine-supplemented groups. In particular, the mIL-23, mIFNγ+mIL-2+mTNFα, mIL-15+mIL-18, R848+TDB, and R848+c-di-GMP supplemented groups showed a significant increase in cell expansion ($p < 0.001$; **Figure 2E**, **Supplementary Figure 3E**). In the mIFNα-treated group, CD335 $(NKp46)^+CD27^+$ cells, known as memory-like NK cells, expanded as well as increased in number (**Figure 2F**, **Supplementary Figure 3F**). The populations of DCs ($CD11c^+$MHC II^+) and MΦs ($CD11b^+F4/80^+$) in this study were not significant (data not shown).

To validate FMDV O Ag-specific T cell response and amplification of memory T cell response due to Ag re-stimulation, $IFN\gamma^{pos}CD4^+$ T cells and $IFN\gamma^{pos}CD8^+$ T cells percentages were compared via flow cytometric analysis of purified T cells from pre- or post- Ag injected mouse PEC with or without Ag treatment. The percentage of $IFN\gamma^{pos}CD4^+$ T cells was significantly increased by Ag treatment, and these Ag-specific T cell responses were remarkably amplified by Ag re-stimulation in the post- Ag injected group ($p < 0.001$; **Supplementary Figure 1A**). A similar trend was detected in the percentage of $IFN\gamma^{pos}CD8^+$ T cells ($p < 0.001$, **Supplementary Figure 1B**). ELISA results demonstrated that IFNγ expression in T cell culture supernatants was also significantly increased by Ag treatment ($p < 0.001$), and the production of IFNγ was significantly enhanced by Ag re-stimulation ($p < 0.001$, **Supplementary Figure 1C**).

Administration of Individual PRR Ligands, Alone or in Combination, Does Not Elicit LDH Release-Related Cytotoxicity in Bovine- and Porcine-Derived PBMCs

LDH release was examined to observe cytotoxicity due to the PRR ligands, gels, and saponins in bovine-derived PBMCs. Low cytotoxicity was observed in all treated cells (**Supplementary Figure 2A**). LDH release following treatment of bovine-derived PBMCs with individual PRR ligands and a vaccine-adjuvant mixture of oil+gel+saponin, is shown (**Supplementary Figure 2B**). At this time, no cytotoxicity due to the adjuvant mixture was observed, compared with control cells. Moreover, cells treated with either the PRR ligand alone (**Supplementary Figure 2C**) or PRR ligands in combination with oil+gel+saponin mixture also exhibited low LDH release levels (**Supplementary Figure 2D**).

When porcine-derived PBMCs treated with individual PRR ligands, or with individual PRR ligands combined with the oil+gel+saponin mixture, were assessed for LDH release, no toxicity was observed at the adjuvant concentrations used in this study, which was similar to the results obtained for bovine-derived PBMCs (**Supplementary Figures 2E,F**). A similar pattern was observed when porcine-derived PBMCs treated with either the PRR ligand alone or PRR ligands in combination with oil+gel+saponin mixture also exhibited low LDH release levels (**Supplementary Figures 2G,H**).

Treatment With Individual PRR Ligands, Alone or in Combination, Promotes Cell Proliferation and Initiates an Immune Response in Bovine- and Porcine-Derived PBMCs

When bovine-derived PBMCs were treated with individual PRR ligands alone (**Figure 3A**) or in combination with oil+gel+saponin mix (**Figure 3B**), cell proliferation was

observed at 96 h via BrdU incorporation, indicating that cell proliferation was increased in all PRR ligand-treated groups compared to the control group. Among these, Curdlan, TDB, c-di-GMP, R848, and Furfurman, in particular, showed the greatest effect. In addition, treatment of PBMCs with PRR ligand combinations or individual PRR ligands combined with the oil+gel+saponin mixture increased cell proliferation in all experimental groups, compared with that of the control group. However, the increase in cell proliferation was relatively lower when oil+gel+saponin was not added (W/O). The results of cell proliferation following treatment with PRR ligand combination with or without oil+gel+saponin are shown (**Figures 3C,D**). While cell proliferation was increased in all treatment groups compared to the control group, the experimental groups treated with R848+TDB, Furfurman+TDB, Curdlan+c-di-GMP, and TDB+c-di-GMP, in particular, showed the highest values.

The results of cell proliferation in porcine-derived PBMCs at 96 h following treatment with individual PRR ligands and cytokines are shown (**Figure 3E**). Increased cell proliferation was observed in all groups treated with the PRR ligands and cytokines, among which TDB and c-di-GMP, in particular, showed the greatest effect. Treatment with individual PRR ligands and cytokines combined with oil+gel+saponin increased cell proliferation in all experimental groups, compared with the control group, as was observed in bovine-derived PBMCs. However, the extent of the increase in cell proliferation was relatively lower when oil+gel+saponin was not added (**Figure 3F**). Cell proliferation following treatment with PRR

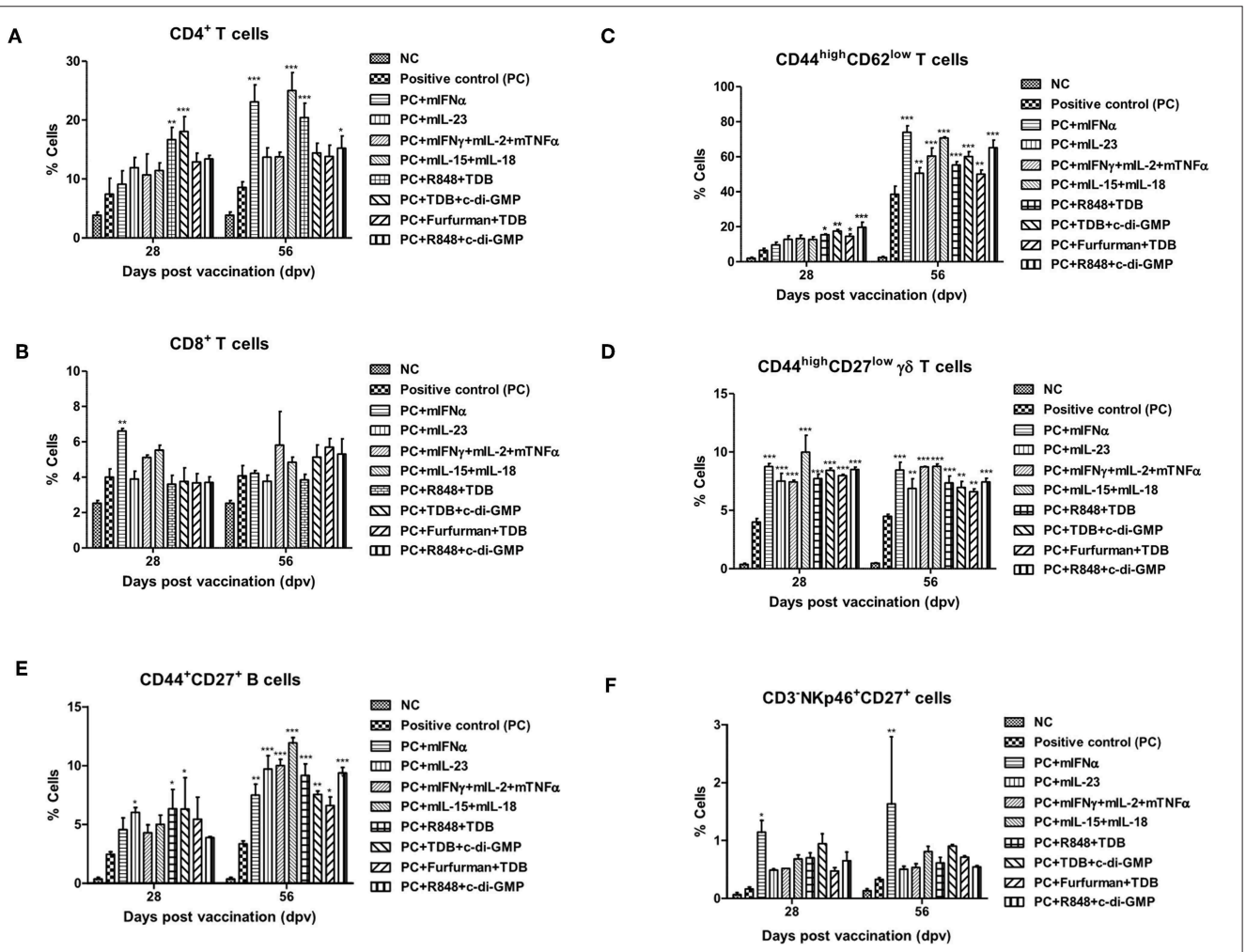

FIGURE 2 | PRR ligands and cytokines promote the expansion of memory immune cells C57BL/6 mice were administered either a combination of PRR ligands or cytokines with the vaccine based on the vaccine composition of the positive control group. The PRR ligands and cytokines used in the experiment and the vaccination method are summarized in **Figure 1**. Peritoneal exudate cells (PEC) sampling was performed at 28 and 56 dpv for the flow cytometric assay. PEC was immunostained with fluorochrome-conjugated Abs to CD3, CD4, CD8a, CD44, CD62L, CD27, γδ TCR, CD335 (NKp46), CD11c, Anti-MHC Class II, CD11b, and anti-F4/80. Data were acquired by flow cytometry and analyzed by FlowJo software vX 0.7. **(A–E)** represent the expansion of immune cells; **(A)** $CD4^+$ T cells; **(B)** $CD8^+$ T cells; **(C)** $CD44^{high}$ $CD62^{low}$ T cells; **(D)** $CD44^{high}$ $CD27^{low}$ γδ T cells; **(E)** $CD44^+CD27^+$ B cells; **(F)** CD335 $(NKp46)^+CD27^+$ cells. The data are the mean ± SEM of triplicate measurements; statistical analyses were performed using two-way ANOVA with Bonferroni correction; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ligand combinations, with or without oil+gel+saponin, is shown (**Figures 3F,G**). Compared with the control group, increased cell proliferation was observed in all treatment groups, and, in particular, the experimental groups treated with R848+TDB, Furfurman+TDB, and TDB+c-di-GMP showed the highest values. On the other hand, cross-species comparison of cell proliferation revealed that cattle showed higher cell proliferation than pigs.

Mincle, STING, Dectin-1/2, and TLR-7/8 Signaling Amplify Robust, Long-Lasting Memory Responses by Inducing Cellular Immune Responses in the Early Stages After Vaccination in Cattle and Pigs

To evaluate the effect of the adjuvants and the memory response mediated by the PRR ligands in farm-raised cattle, R848+TDB and Curdlan+c-di-GMP (both of which showed a significant effect in the PRR ligand-screening experiment using bovine PBMCs) were applied to an animal experiment using the strategy shown in **Figure 4A**. At 28 dpv after the first vaccination, Ab titers were determined by SP O ELISA, and significantly higher antibody titers were observed in the experimental groups treated with R848+TDB ($p < 0.05$) and Curdlan+c-di-GMP ($p < 0.01$) than in the positive control group; the antibody titers were maintained at high levels, up to 168 dpv after boosting ($p < 0.05$ and $p < 0.001$, respectively) (**Figure 4B**). In addition, when the VN titer was examined, the titer was significantly higher in the PRR ligand-treated groups than in the control group from 14 dpv ($p < 0.01$), and the titer was maintained at very high levels even until 140 dpv ($p < 0.001$) (**Figure 4C**).

To investigate the effect of the adjuvants as well as the memory response mediated by the PRR ligands in farm-raised pigs, R848+TDB, Furfurman+TDB, and TDB+c-di-GMP (both of which showed a significant effect in the PRR ligand-screening experiment using porcine PBMCs) were applied to the animal experiment (**Figure 5A**). To determine the antibody titers induced by vaccination, SP O ELISA was performed using porcine serum. Antibody titers were significantly increased ($p < 0.001$) in the TDB+c-di-GMP-treated group at 14 dpv compared to the positive control group ($p < 0.001$), and the antibody titers were drastically increased ($p < 0.001$) in all groups treated with the PRR ligands at 28 dpv (**Figure 5B**). In particular, antibody titers were maintained at significantly higher levels (up to 84 dpv, $p < 0.01$) in the TDB+c-di-GMP-treated group compared to the control group. Furthermore, when the VN titer was determined, significantly higher neutralizing antibody titers were observed in the TDB+c-di-GMP ($p < 0.001$) and R848+TDB groups ($p < 0.01$) at 14 dpv compared to the positive control group. Antibody titers were also drastically increased ($p < 0.001$) in all groups treated with PRR ligands at 28 dpv ($p < 0.001$). In particular, antibody titers were maintained at high levels in the TDB+c-di-GMP-treated group (up to 84 dpv, $p < 0.001$), while the R848+TDB-treated group showed an excellent immune-boosting effect from the early (14 dpv) to middle (42 dpv) stages ($p < 0.01$ and $p < 0.001$, respectively) post vaccination, which tended to slightly decrease thereafter. In contrast, in the Furfurman+TDB group, the neutralizing antibody titers increased somewhat slowly in the early stage (up to 14 dpv) but drastically increased thereafter from 28 to 84 dpv compared to the control group ($p < 0.01$ and $p < 0.001$, respectively) (**Figure 5C**). However, neutralizing antibody titers increased more rapidly in cattle than in pigs, and even after boosting, neutralizing antibody titers tended to remain at higher levels in cattle than in pigs.

The above results indicate that R848 (TLR-7/8)+TDB (Mincle) and Curdlan (Dectin-1)+c-di-GMP (STING) specifically increased the cellular immune response and induced long-lasting memory responses in cattle. Similar reactions were also observed in TDB (Mincle)+c-di-GMP (STING), R848 (TLR-7/8)+TDB (Mincle), and Furfurman (Dectin-2)+TDB (Mincle).

DISCUSSION

FMD is classified as an acute infectious disease in cattle and pigs. It is asymptomatic in small ruminants, which can cause persistent infections, making it difficult to eradicate (17). Currently, vaccination policies are being implemented in Korea and other countries facing FMD epidemics, and, in the event of an outbreak, large-scale vaccine production is necessary to prepare for nationwide vaccination.

The innate immune response acts as the host's first line of defense against invading pathogens. Innate immune cells, particularly dendritic cells (DCs) and macrophages (MΦs), sense external microorganisms, which are recognized by PRRs via pathogen-associated molecular patterns (PAMPs) associated with the microrganisms. PRRs not only mediate the activation of innate immune cells in the presence of a danger signal, such as infection, but they also directly regulate adaptive immune responses (21). Although adjuvants have traditionally been used to boost the immunogenicity of vaccines, little is known about the host reactivity and precise mechanisms related to adjuvants contained in animal vaccines targeted toward different livestock species.

Recently, Chen et al. (9) reported that an adjuvant mixed with MDP (NOD-2 ligand), MPL (TLR-4 agonist), and β-glucan (TLR-2, TLR-4, and TLR-6 ligands) improved immune response and protection in pigs when used in combination with FMD vaccines. However, the immunological mechanism underlying the improved immunity has not yet been clearly understood. Martinez-Lopez et al. (22) reported that Mincle plays a key signaling role in microbiota sensing by stimulating the secretion of IL-23p19 and IL-6 via the Mincle-Syk axis, thereby regulating secretion of IL-17 and IL-22 from Th17 cells. In addition, Dectin-1/2 has also been reported to regulate the immune response against *Mycobacterium tuberculosis* infection by stimulating secretion of IL-23 through Syk-CARD9 signaling and the secretion of IL-17 from Th17 in DCs and MΦs (23, 24). STING is known to be involved in the antiviral activity mediated by cytosolic DNA sensing (25) and type I IFN expression through the cGAS-cGAMP-STING pathway (26, 27). TLR-7/8, the most well-known component of the TLR pathway, has also

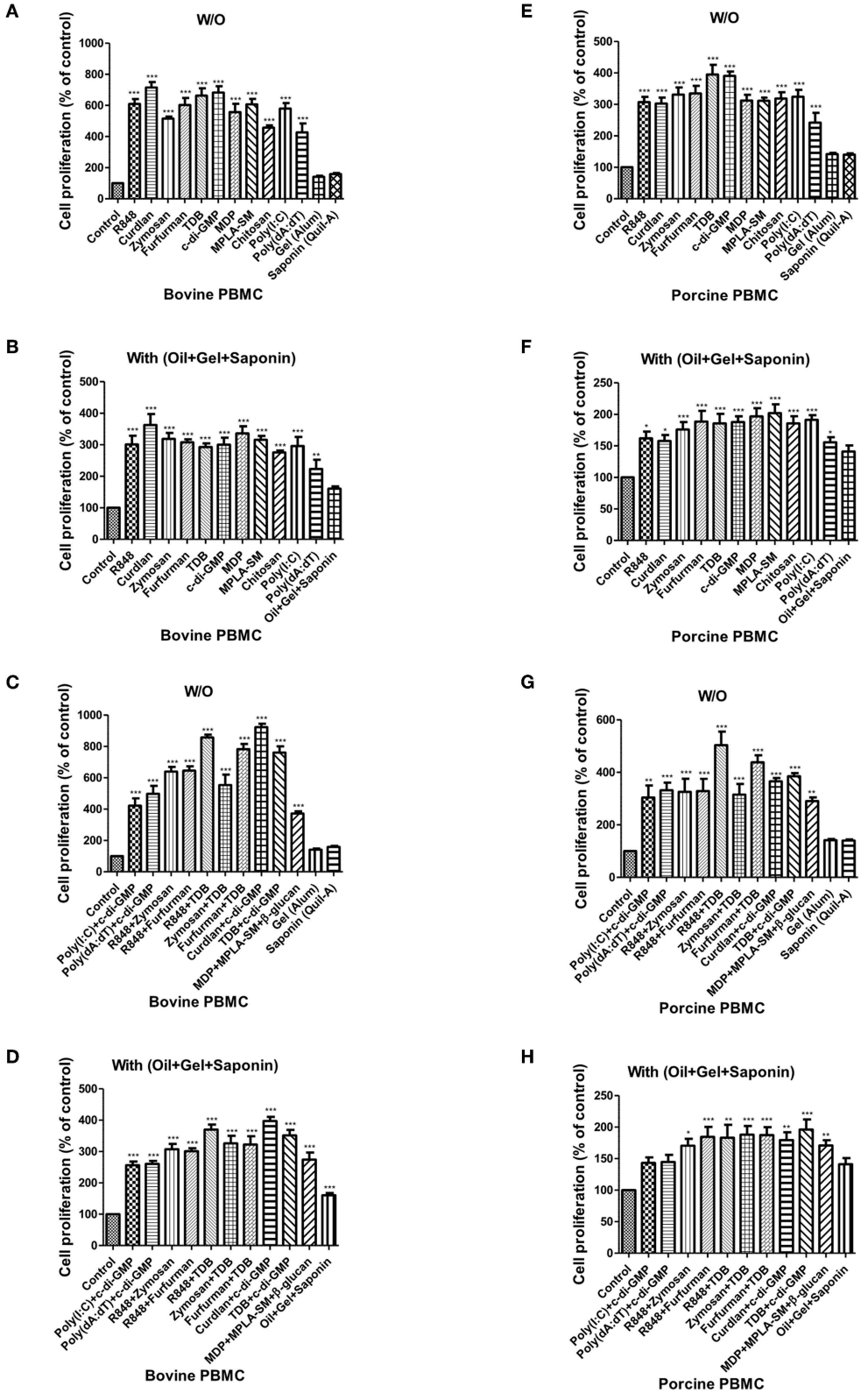

FIGURE 3 | PRR ligand-induced bovine and porcine PBMC proliferation, as assessed by a BrdU cell proliferation kit. Bovine and porcine PBMCs were co-incubated with either PRR ligands alone or a combination of PRR ligands or a mixture of oil, gel, and saponin. The PRR ligands used in the experiment were as follows: R848

(Continued)

FIGURE 3 | (TLR-7/8 agonist), Curdlan (Dectin-1 agonist), Zymosan (Dectin-2/TLR-2 agonist), Furfurman (Dectin-2 agonist), TDB (Mincle agonist), c-di-GMP (STING agonist), MDP (NOD-2 agonist), MPLA-SM (TLR-4 agonist), chitosan (NLRP3 inflammasome inducer and MR agonist), poly(I:C) (TLR-3/MDA-5 agonist), poly(dA:dT), RIG-1/CDS agonist, and AIM2 inflammasome inducer. Gel alone, saponin alone, and a mixture of oil, gel, and saponin were also tested for comparison. At specific time points (96 h) after coincubation, cell proliferation was tested using a BrdU ELISA kit. **(A–D)** represent *in vitro* cell proliferation induced by the PRRs in bovine PBMCs; **(A)** PRR ligands alone; **(B)** PRR ligands with a mixture of oil, gel, and saponin; **(C)** combination of PRR ligands; **(D)** combination of PRR ligands with a mixture of oil, gel, and saponin. **(E–H)** represent *in vitro* cell proliferation induced by the PRRs in porcine PBMCs; **(E)** PRR ligands alone; **(F)** PRR ligands with a mixture of oil, gel, and saponin; **(G)** combination of PRR ligands; **(H)** combination of PRR ligands with a mixture of oil, gel, and saponin. The data are the mean ± SEM of triplicate measurements ($n = 6$); statistical analyses were performed using one-way ANOVA with Tukey's post-test; $^{*}p < 0.05$, $^{**}p < 0.01$, *and* $^{***}p < 0.001$.

been reported to inhibit viral replication during viral infection through IFNα secretion as well as enhance mucosal immunity and systemic immune response (28, 29). However, little research had previously been conducted on the applicability of PRR ligands as an adjuvant for FMD vaccines.

A previous study by our group confirmed that the FMDV Ag-mediated activation of DCs and MΦs is induced by the stimulation of specific PRRs such as Mincle, STING, Dectin-1/2, and TLR-7/8 in mice. In addition, cytokines, such as IL-23 and IFNα (which are directly induced by FMDV Ag and expressed in DCs and MΦs), as well as the ligands that can stimulate associated PRRs were confirmed to significantly improve the protective effect of the FMD experimental vaccines against FMDV when used as a FMD vaccine adjuvant in a host.

Based on these results, this study aimed to monitor the memory response mediated by the PRR ligands and cytokines when used as an FMD vaccine adjuvant. This would likely enable development of FMD vaccine adjuvants, and vaccine compositions containing these adjuvants, which are optimized for bovine and porcine livestock species.

In mice, injections of various PRR ligands and cytokines alone or in combination as an adjuvant induced higher antibody titers in all experimental groups compared to the positive control group after a single vaccination. Although the short *in vivo* persistence of IFNα has been mentioned as a disadvantage in several papers (30, 31), the present study demonstrated that injection of IFNα as an adjuvant generated high antibody titers and that the resulting immunity persisted up to 56 dpv. The IL-23-treated group (in which the cellular immune response and host protective effect were previously demonstrated to be significantly improved), the group treated with IFNγ+IL-2+TNFα (which are both involved in T cell activation and T cell-mediated cellular/humoral immune responses) and the group treated with IL-15+IL-18 (which are involved in mucosal immunity) all continuously maintained high antibody titers. The PRR ligand-treated groups also showed similar patterns, confirming that these adjuvants can effectively induce memory responses.

When the effect of vaccination itself on weight change was examined, a slight weight loss was observed in the IFNγ+IL-2+TNFα combination group at 7 dpv, but no significant difference was found. This may be due to the slightly higher dose of the recombinant cytokines (15 μg in total) in this group compared to that of the other groups (5–10 μg in total). Notably, Ag-specific T cell response was significantly amplified by Ag re-stimulation. Inclusion of PRR ligands and cytokines as adjuvants promoted memory T cells, memory γδ T cells, memory B cells, and memory-like NK cell expansion, thereby effectively enhancing cellular immunity and humoral immunity. The expansion of these memory cells will play an important role in host defense by enabling a more rapid and strong response to the pathogen during FMDV infection.

Taken together, the induction of robust memory responses and expansion of memory cells by PRR ligands and cytokines resulted in a complete protective effect against FMDV infection in all experimental groups (**Figures 1**, **2**).

Zhou et al. (8) reported that R848 and poly(I:C) injected in combination with $Al(OH)_3$ as an FMD vaccine adjuvant enhanced the immune response in mice. Additionally, Du et al. (32) recently showed that CVC 1302 (MDP, MPL, and β-glucan) could be added to commercially available inactivated FMDV (serotype O) vaccines as an adjuvant-induced, long-term humoral immunity in mice through the stimulation of T follicular helper cells and the germinal center response. However, these studies were performed using specific PRR ligands, and a study to investigate the protective effects of these substances against actual FMDV infection in mice had not been previously conducted. Therefore, the results of the present study, which investigated the effects of a wide range of PRR ligands and cytokines on the induction of memory response and ability to defend against FMDV (serotype O) infection, can be interpreted as a highly efficient adjuvant screening system. This screening system may be of us in a pre-animal experimental step targeting specific animal species, such as cattle and pigs, and thus can also be used to provide basic data for developing FMD vaccines via a new strategy.

Pigs are known to have lower persistence and efficacy in their immune responses compared to cattle (7). To propose FMD vaccine compositions with superior efficacy optimized for each livestock species and to overcome the immunogenicity gap between the two species, screening was performed to identify adjuvants that could stimulate the immune response specifically in cattle and pigs. In order to facilitate screening, PBMCs isolated from the whole blood of cattle and pigs were treated with various PRR ligands and cytokines alone or in combination, and the LDH release-related cell cytotoxicity and cell proliferation were examined. PBMCs consist of lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells. PBMCs are broadly used in the fields of immunology, infectious disease, vaccine development, and transplant immunology, among others. Therefore, bovine and porcine PBMCs are useful model systems for the study of FMD vaccine and adjuvants. No cytotoxicity was observed in any of the adjuvant

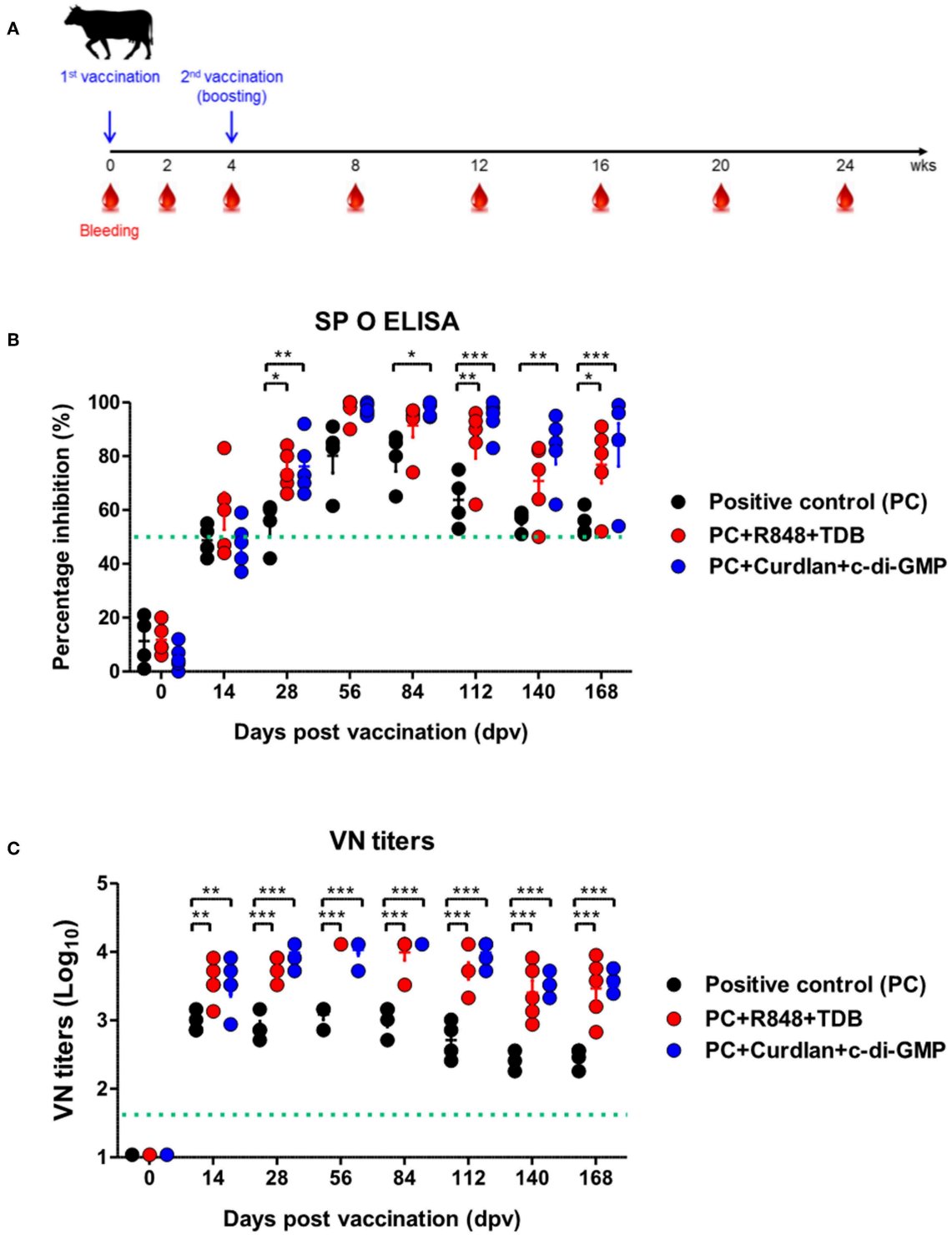

FIGURE 4 | PRR ligand-mediated long-lasting memory response in cattle. Cattle were administered either a combination of R848 (TLR-7/8 agonist) and TDB (Mincle agonist) or Curdlan (Dectin-1 agonist) and c-di-GMP (STING agonist) with the vaccine, based on the vaccine composition of the positive control group. A positive control group of cattle received 15 μg (1 dose for cattle use) of O/TWN/97-R Ag, ISA 206 (50%, w/w), 10% $Al(OH)_3$, and 150 μg Quil-A without PRR ligands. The vaccination was performed twice at a 28 days interval, and 1 ml vaccine (1 dose) was injected via the deep intramuscular route on the necks of the animals. Blood samples were collected at 0, 14, 28, 56, 84, 112, 140, and 168 dpv from the cattle for the serological assays. **(A–C)** represent **(A)** the strategy for this study; **(B)** antibody titers by SP O ELISA; **(C)** virus-neutralizing antibody titers. The data are the mean ± SEM of triplicate measurements; statistical analyses were performed using two-way ANOVA with Bonferroni correction; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

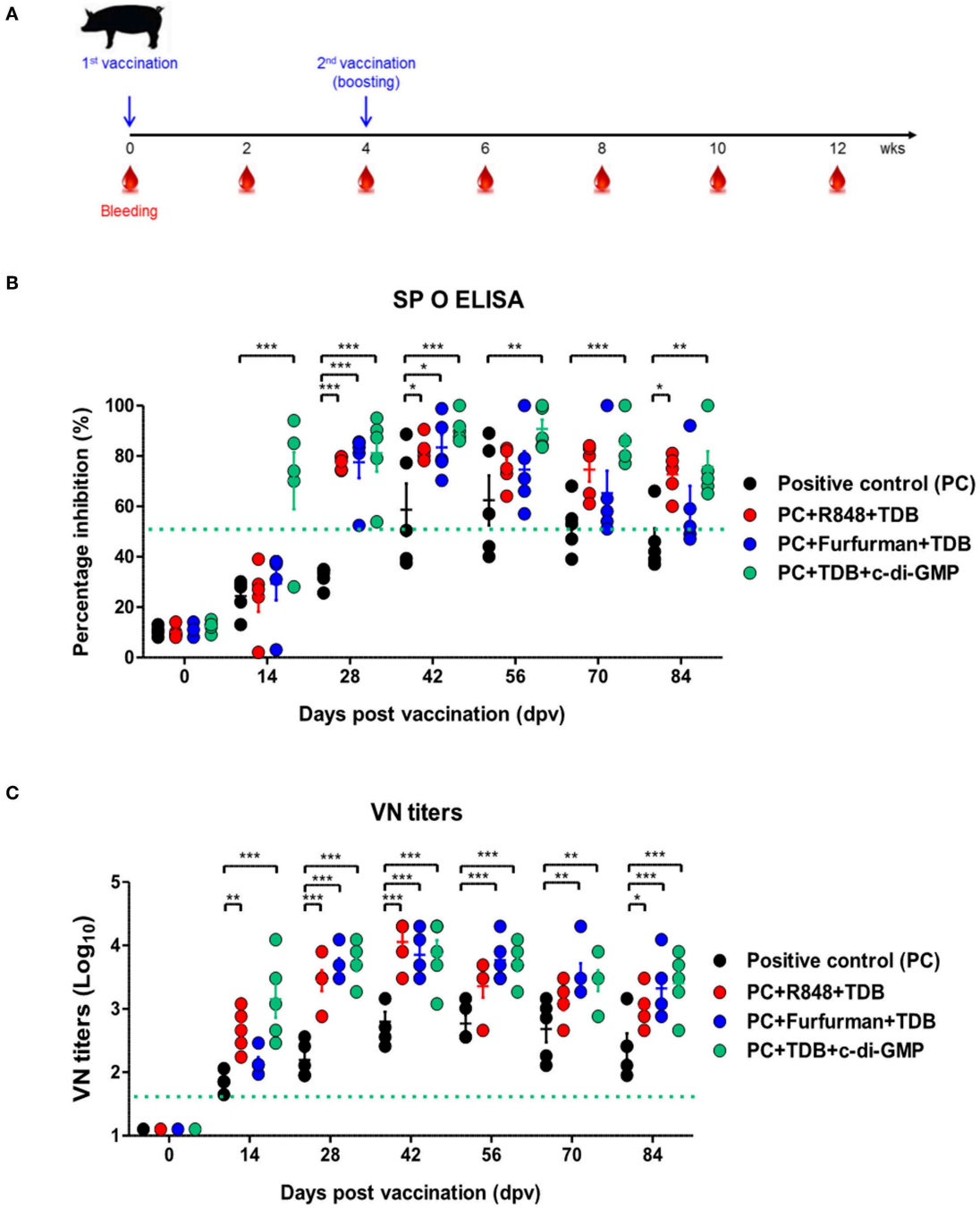

FIGURE 5 | PRR ligand-mediated long-lasting memory response in pigs. Pigs were administered a combination of R848 (TLR-7/8 agonist) and TDB (Mincle agonist) or Furfurman (Dectin-2 agonist) and TDB (Mincle agonist) or TDB (Mincle agonist) and c-di-GMP (STING agonist) with the vaccine based on the vaccine composition of the positive control group. The positive control group of pigs received 15 μg (1 dose for pig use) of O/TWN/97-R Ag, ISA 206 (50%, w/w), 10% $Al(OH)_3$, and 150 μg Quil-A without PRR ligands. The vaccination was performed twice at a 28 days interval, and 1 ml vaccine (1 dose) was injected via the deep intramuscular route on the necks of the animals. Blood samples were collected at 0, 14, 28, 42, 56, 70, and 84 dpv from the pigs for the serological assays. **(A–C)** represent **(A)** the strategy for this study; **(B)** antibody titers by SP O ELISA; **(C)** virus-neutralizing antibody titers. The data are the mean ± SEM of triplicate measurements; statistical analyses were performed using two-way ANOVA with Bonferroni correction; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

concentrations used in this study, indicating that these adjuvants are safe to administer to a host. These adjuvants significantly increased cell proliferation compared to the control group, and R848+TDB and Curdlan+c-di-GMP were effective in cattle, while R848+TDB, Furfurman+TDB, and TDB+c-di-GMP were effective in pigs. These PRR ligands mediated cell proliferation in bovine and porcine PBMCs, which is expected to simultaneously stimulate various immune cells

to induce cellular immune responses more efficiently. In the cross-species comparison in particular, the immune response in bovine-derived PBMCs was significantly higher than that of porcine-derived PBMCs for most adjuvants, suggesting that bovine immune cells are more sensitive to external stimuli than porcine immune cells. Our group previously identified the fundamental difference in the FMDV Ag-mediated immune response between bovine and porcine immune cells. In the previous study, even though the Ag was a porcinophilic virus, FMDV (serotype O) Ag stimulated remarkably higher cell proliferation in bovine immune cells (PBMCs, lymphocytes, monocytes, and T cells) than in porcine immune cells. The discovery of this difference may explain the phenomenon of the lower immunogenicity observed in pigs, as compared to cattle, and suggest a key clue to overcoming this problem. In addition, based on the results of treating PBMCs with a mixture of ISA 206, $Al(OH)_3$, and saponin, it is expected that when these adjuvants are injected as an actual vaccine component, the oil emulsion will allow the Ag and adjuvant to be released slowly *in vivo*, thus enabling continuous stimulation of immune responses (**Supplementary Figure 1**, **Figure 3**).

Based on the findings from the screening of PRR ligands and cytokines in PBMCs isolated from each species, experimental vaccines were created, and their immunogenicity was tested in farm raised cattle and pigs. The results showed that the neutralizing antibody titers significantly increased in both the bovine and porcine groups treated with species-specific PRR ligands at 14 dpv after the first vaccination compared to the positive control group. Long-lasting immune responses were also observed after the second vaccination (boosting). The high level of titers of neutralizing antibodies confirmed in both the cattle and pigs can be interpreted to mean that the combination of ligands, such as Mincle, STING, TLR-7/8, and Dectin-1, stimulates extrinsic and intrinsic pathways simultaneously to effectively initiate innate and cellular immune responses and activate various kinase pathways, effector molecules, and transcription factors to induce cytokine secretion. The cellular immune response is there by enhanced, and the humoral immune response is strongly induced as well. In addition, the second booster vaccination at 28 dpv showed to effectively induce "recall stimulation" among the immune cells stimulated by the first vaccination, and it also plays a role in maintaining long-lasting immune responses. The combination of the Mincle+STING ligands in pigs specifically resulted in an excellent increase of the antibody titers from 14 dpv, which is expected to help overcome the disadvantages of commercial vaccines for pigs (**Figures 4**, **5**). In addition, further studies on the efficacy of cytokine adjuvants (which showed a strong memory response-inducing effect and a protective effect in mice) should be conducted on the target animals (cattle and pigs), and the economic feasibility of the vaccine with adjuvant addition should be considered in the future.

In summary, the novel FMD vaccine platform utilizing the Mincle, STING, Dectin-1/2, and TLR-7/8 ligands as adjuvants is expected to open the door to a new era in the field of FMD prevention and treatment.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethics Committee of the Animal and Plant Quarantine Agency.

AUTHOR CONTRIBUTIONS

ML designed the research, performed and analyzed overall experiments, and wrote the manuscript. HJ supported overall experiment. SS supported animal experiment. S-MK advised experiments. BK advised the study and reviewed the manuscript. HS provide whole blood from cattle and pigs for *in vitro* study. J-HP supervised and 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.2019.02509/full#supplementary-material

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Conflict of Interest: The 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.

frontiers in Immunology

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Heterologous Immunity: Role in Natural and Vaccine-Induced Resistance to Infections

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The central paradigm of vaccination is to generate resistance to infection by a specific pathogen when the vacinee is re-exposed to that pathogen. This paradigm is based on two fundamental characteristics of the adaptive immune system, specificity and memory. These characteristics come from the clonal specificity of T and B cells and the long-term survival of previously-encountered memory cells which can rapidly and specifically expand upon re-exposure to the same specific antigen. However, there is an increasing awareness of the concept, as well as experimental documentation of, heterologous immunity and cross-reactivity of adaptive immune lymphocytes in protection from infection. This awareness is supported by a number of human epidemiological studies in vaccine recipients and/or individuals naturally-resistant to certain infections, as well as studies in mouse models of infections, and indeed theoretical considerations regarding the disproportional repertoire of available T and B cell clonotypes compared to antigenic epitopes found on pathogens. Heterologous immunity can broaden the protective outcomes of vaccinations, and natural resistance to infections. Besides exogenous microbes/pathogens and/or vaccines, endogenous microbiota can also impact the outcomes of an infection and/or vaccination through heterologous immunity. Moreover, utilization of viral and/or bacterial vaccine vectors, capable of inducing heterologous immunity may also influence the natural course of many infections/diseases. This review article will briefly discuss these implications and redress the central dogma of specificity in the immune system.

Keywords: heterologous (non-specific) effects of vaccines, heterologous immunity, T cells, antibody, innate and adaptive immune response

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INTRODUCTION

Studies in humans and mouse models have clearly demonstrated that exposure or infection with one pathogen can induce and/or modify the immune response against another unrelated pathogen. This is what's defined as heterologous immunity (1, 2). The ability of an individual to respond to a pathogen is influenced by its exposure history to a significant extent, both by pathogenic microbes and commensals (microbiota) (2). Heterologous immunity could boost or weaken protective immunity against a pathogen, and/or induce severe immunopathology or tolerance against self-antigens. Therefore, there must be a delicate balance between protective immunity and immunopathology, and heterologous immunity can play an important role in tilting this balance. Heterologous adaptive immunity was initially thought to be due to high levels of amino acid

sequence similarities in T cell and B cell epitopes among antigens of different pathogens, but has now broadened in scope with the realization of the highly cross-reactive nature of adaptive immune lymphocytes (3). With the expanding understanding of heterologous immunity, there is a need to re-think and re-examine its impact on vaccines, resistance to infections, protective vs. detrimental immunity, as well as autoimmunity. In this article, I will briefly review heterologous immunity, and its impact on natural immunity to infections, vaccine vectors and vaccine-mediated protection by describing, (1) the realm of cross-reactive adaptive lymphocytes, (2) evidence of cross-reactivity from vaccine studies, (3) animal and human model experiments to demonstrate cross-reactivity between a broad range of pathogens, (4) possible influence of cross-reactivity on natural resistance to infections, (5) role of microbiota in heterologous immunity, (6) vaccine vectors and heterologous immunity, and (7) discussion and future prospects. In addition to cross-reactive lymphocytes, a network of cytokines, regulatory cells and trained innate immune cells contribute to manifesting heterologous immunity (4, 5). However, this article is more focused on cross-reactive adaptive immune lymphocytes.

THE REALM OF CROSS-REACTIVE ADAPTIVE CELLULAR IMMUNITY

Heterologous immunity is the induction of an immune response to an unrelated pathogen/antigen upon exposure to a different pathogen/antigen. Conceptually, cross-reactivity (or poly-specificity) of lymphocytes in antigen (or epitope) recognition is foundational to heterologous adaptive immunity (**Figure 1**).

The essence of clonal selection theory of T cells is based on: one epitope specificity-one T cell clonotype, which defines and contributes to the high specificity of adaptive cellular immune responses and differentiates them from innate lymphocytes that rely on broad pattern recognition (6, 7). Accordingly, it is assumed that T cells bearing a T cell receptor (TCR) for a specific peptide epitope [∼9 amino acids (aa) for CD8 and ∼11 aa for CD4] emerge in an individual long before they are exposed to the corresponding foreign antigen through random variable (V), diversity (D) and joining (J) (VDJ) regions' recombination of TCR α and β chains and permutations of α and β chain heterodimers, as well as rounds of positive and negative selection in the thymus.

The high specificity and the apparent stringency of the specific interaction of peptide bound with major histocompatibility complex and T cell receptor (p-MHC:TCR) complexes were demonstrated by X-Ray crystallography studies (8). These structural studies were followed by a wide range of flow cytometry studies encompassing p-MHC tetramer-based detection of T cells with stringent peptide specificity, supporting and perpetuating the one specificity-one clonotype theory (9). Observations contradicting this theory were few and often not well-documented in literature. Technical limitations in clearly defining and characterizing cross-reactivity (or heterologous immunity) at the molecular level and reliance on empirical cellular methodologies could partially explain an apparent scarcity of reporting of cross-reactivity in experimental systems. Regardless, they have substantial clinical implications in vaccine and immunotherapy applications.

It has been predicted that high affinity would reflect a better quality immune response, and indeed, in viral immunology the demonstration of specific p-MHC binding T cells was suggested to correlate to protection from infection or viral clearance (10, 11). Nevertheless, the relationship between the affinity of the TCR and p-MHC, and the subsequent immune response is not direct and may lead to unexpected immune responses and undesired consequences. Cancer immunotherapy has anticipated that an enhanced therapeutic effect might occur when TCR affinity is increased in TCR-based therapeutics such as chimeric antigen receptors bearing T cells (CAR-T) cells and T cell adoptive transfer. However, clinical trials using high affinity T cell adoptive transfer against the melanoma associated antigen-3 (MAGE-3), resulted in off-target cross-reactivity against a cardiac-associated Titin antigen and fatality (12). There was only 55% homology between the MAGE-3 peptide and Titin-derived peptide, but X-ray studies demonstrated similarity in p-MHC complexes, which formed the basis for the observed cross-reactivity (13, 14). Thus, TCR-pMHC affinity alone may not predict the efficiency of the immune response or clinical success in both vaccine and T-cell adoptive transfer based therapeutics.

Physiologically, it is rather puzzling that the T cells are believed to be individually highly specific, and yet expected to collectively respond to a huge number of foreign antigens in a host to provide protective immunity against a vast number of pathogens of various classes during lifelong exposures. Also, a logistic problem that exists in the available functional T cell repertoire in an individual is the limited number of T cell clonotypes, i.e., $\sim 10^6$ in mice and $\sim 10^8$ in humans, which would severely limit the extent of immune responses generated, especially since at least an estimated $>10^{15}$ peptides must be effectively responded against, in a host's lifetime (3, 15, 16). Based on mathematical calculation of this disparity, it has been hypothesized that each T cell clonotype needs to recognize $\sim 10^6$ different p-MHC combinations in order for adaptive cellular immune system to be effective (3, 17). Therefore, revisiting of the concept of clonal selection and one-specificity-one T cell is warranted. Classical $CD8^+$ and $CD4^+$ T cells recognize peptide epitopes of 9–16 aa in the context of MHC class I and class II molecules, respectively. However, in their recognition, the TCR only contacts 3–5 aa of the peptide bound to the MHC molecules, and at minimum, 4 aa long peptides have been shown to stimulate T cell activation (15, 18). TCR recognizing a p-MHC complex glides over a relatively flat surface, culminating with favorable interaction between the complementarity determining region (CDR) of the TCR and 3–4 amino acids of the MHC bound peptide. Thereafter, the TCR binds with the p-MHC complex generally with short range weak intermolecular bonds, i.e., van der Waal's forces

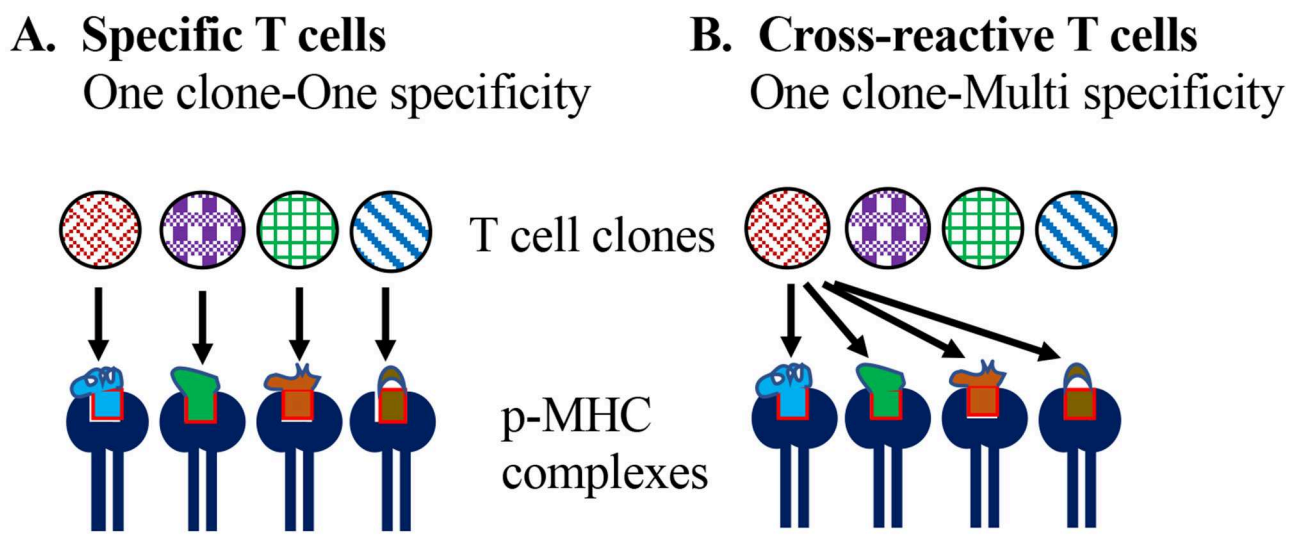

FIGURE 1 | Specificity vs. cross-reactivity of T cells. **(A)** According to the one-clone-one-specificity model, individual clones of T cells recognize one specific peptide epitope in the context of self MHC molecules, and do not recognize or get stimulated with any other peptide. **(B)** Model of T cell cross-reactivity implies an individual T cell clone can recognize multiple peptide epitopes in the context of the self MHC molecule.

(2). TCR:p-MHC binding is characterized with low numbers of hydrogen bonds and almost no covalent bonds, resulting in low affinities of interaction (19). Multimerization of p-MHC provides the strength of a T cell recognizing p-MHC on antigen presenting cells (APCs), that results in activation thresholds (20). In addition to the low-affinity binding, the complementarity determining region (CDR) of the TCR demonstrates structural rearrangements and plasticity in conformation in binding to the p-MHC complex, as well as variations in angles of docking onto p-MHC, and conformational shifts in both peptide and MHC of the p-MHC complex. All these changes result in thermodynamic and physicochemical mechanisms for cross-reactivity of the TCR (15). Furthermore, structural degeneracy of amino acids in the peptide bound to MHC supports the cross-reactive recognition by the TCR (15). These include the physicochemical properties of the amino acids that provide the hydrophobic characteristics for Van der Waal's interaction between TCR and p-MHC instead of the strict identity of the amino acid. These mechanisms, along with the minimal contact requirement of the TCR on p-MHC, i.e., 3–5 aa, would provide conditions conducive for cross-reactivity. In addition, T cells are positively selected in the thymus based on a limited selection of self-peptide epitopes bound to MHC molecules, and yet result in T cells recognizing a wide variety of previously unseen foreign peptides. This selection process, in addition to the structural considerations mentioned earlier, add to an absolute inherent requirement for T cells to be cross-reactive (21). Based on these features, it is understandably a very difficult task to recognize cross-reactivity using current molecular techniques. Most characterization of cross-reactivity of T cells has been achieved through empirical observations in humans and animal models, using epidemiological studies and cellular techniques.

VACCINES AND EVIDENCE (OR MANIFESTATION) OF HETEROLOGOUS IMMUNITY

Some of the most common and longest-used vaccines in humans are Vaccinia (smallpox vaccine, for smallpox virus), BCG (Bacille Calmette Guerin, for tuberculosis), Measles (for measles virus), OPV (oral polio vaccine), and DTP (for diphtheria, tetanus, and pertussis) (22, 23) (https://www.cdc.gov/vaccines/vpd/index.html). The human use of these vaccines has been very effective in preventing infection with the corresponding pathogen and associated mortality, and has even led to the eradication of smallpox virus infections from the world and polio to the verge of eradication. In addition to the specific effect of these vaccines in preventing the targeted infection, the non-specific or heterologous effect of these vaccines in preventing infections with unrelated pathogens has been recognized through epidemiological studies in human populations (22, 24–27) as described below.

Worldwide mandatory smallpox vaccination during the 1960s and 70s, contributed to the disease being eliminated in 1980 (https://www.cdc.gov/smallpox/history/history.html). Anecdotally, scientists in the nineteenth and early twentieth century reported positive effects of small pox vaccine in multiple diseases other than small pox such as papillomas, chronic skin disorders, eyes, ear, nose and throat disorders, measles, scarlet fever, whooping cough, and syphilis (28). From a cohort of 3,559 individuals in Denmark, it has been found that smallpox vaccination is associated with a reduced risk of infectious disease hospitalization in a high-income setting (29). There has also been some preliminary suggestion that prior immunization with small pox vaccine may provide an individual with some

degree of protection to subsequent Human Immunodeficiency Virus (HIV) infection and that the worldwide termination of smallpox vaccination in 1980 may have partially allowed the HIV epidemic to explode (30). It has been shown in epidemiological studies from Guinea-Bissau and Denmark that smallpox and/or BCG vaccination is associated with a reduced risk of HIV-1 infection in women (27). Using $CCR5^+$ T cells from unvaccinated or smallpox-vaccinated individuals, it was shown that HIV-1 replicated to lower levels in cells from vaccinated individuals compared to unvaccinated people (30). In another study using 97 women with or without a smallpox-vaccine scar, however, CCR5 expression in T cells could not be correlated to protection from HIV-1 infection with vaccination status (31), suggesting that modulation of CCR5 expression apparently does not contribute to the observed protection. It remains to be seen whether heterologous immunity contributed to an apparent protection from HIV-1 infection upon smallpox and/or BCG vaccination, even to a small extent. Overall, these reports suggest a non-specific protective effect of smallpox vaccine on a number of infectious diseases in human population, the mechanisms of which are not clear yet.

BCG vaccination in infants has been shown to reduce infant mortality due to childhood infections such as respiratory infections and sepsis unrelated to tuberculosis in high mortality settings as well as in USA and Europe (24, 32–34). Immunization with BCG has also been shown to reduce the incidence of allergic diseases, and autoimmune/inflammatory diseases such as type I diabetes (T1D) and multiple sclerosis (MS) (35). Removal of the infant BCG vaccination program due to decline in TB cases has been shown to be correlated with increased incidence of respiratory infections, melanoma, lymphoma, atopic dermatitis, asthma etc. (36). Intra-vesicular treatment with BCG has become the mainstream treatment of bladder cancer (37). Along the same lines, studies in animal models have shown that BCG immunization of mice leads to prevention in development of type 1 diabetes (T1D) as well as resistance to vaccinia virus infection (38, 39). Detailed mechanisms of these effects of BCG vaccination are still unclear. However, animal model studies have shown a role of $CD4^+$ T cells, as well as trained innate immunity (39). Role of trained innate immunity in providing heterologous immunity upon BCG vaccination has also been demonstrated in humans (26, 40, 41).

Measles vaccine given to infants has been shown to reduce childhood mortality by infections other than measles by 30–86% in 10 different cohort studies from different countries (42). Similarly, in a study encompassing the years 2002–2014, oral polio vaccine, used widely to eradicate polio has been shown to reduce mortality by 19% (range 5–32%) in children <5 years of age independent of its effect on polio (43). Furthermore, in a randomized clinical trial, vaccination with OPV and BCG at birth demonstrated 32% (0–57%) lower infant mortality than BCG alone (44).

The diphtheria-tetanus-pertussis (DTP) vaccine shows excellent protection against the three targeted diseases, however, it has been shown that mortality in females (but not males) increases from other infectious diseases (45, 46). However, the increased female mortality was only found in children who had received DTP after measles vaccine producing high titers, whereas in subjects receiving measles and/or BCG vaccine after DTP, mortality rate declined substantially (45). It was suggested that immunization with DTP may deregulate the female immune system so that subsequent unrelated infections are fought inefficiently, whereas immunization with BCG or measles vaccine subsequent to DTP may circumvent the harmful effect of DTP (45). Therefore, the non-specific effects of vaccines must be thoroughly studied with respect to sex differences.

Potential mechanisms for heterologous effects of vaccines may include cross-reactivity between shared epitopes of unrelated pathogens, trained immunity in innate cells such as natural killer (NK), natural killer T cells (NKT) and monocytes, modulation of type 1, type 17, regulatory and memory T cells, cytokine responses, and modulation of mean concentration of antibodies as well as cross-reactive antibodies (47). Nevertheless, the exact contribution of each of these potential mechanisms in producing the observed heterologous effects of vaccines remains to be delineated.

HETEROLOGOUS IMMUNITY ACROSS A BROAD RANGE OF PATHOGENS

Heterologous immunity has been shown commonly among closely related pathogens, e.g., different subtypes of influenza A viruses and Dengue viruses, different members of the same family such as within flaviviruses and picornaviruses, and among unrelated pathogens including parasites, protozoa, bacteria, and viruses. It has been suggested that the history of exposure to various microbial infections and the resulting changes in the memory T cell repertoires determine the existence of a cross-reactivity network in each individual, and therefore cross-reactivity against multiple epitopes may be observed in an individual (48).

Heterologous immunity has been experimentally shown in mice by Welsh and Selin, who have reported that some levels of protection against vaccinia virus (VV) infection is obtained in mice that have been earlier exposed to infections with lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), Pichinde virus (PV), or influenza A virus (IAV) (1, 17, 49, 50). Furthermore, LCMV, PV, and MCMV all provide reciprocal cross-reactive immunity in mice (50). Interestingly, influenza virus infection provides cross-protective immunity against VV, but exacerbates infection with LCMV and MCMV. However, VV infection does not cross-protect against any of the tested heterologous pathogens and does not show reciprocal heterologous immunity (51). Our studies have demonstrated unexpected cross-reactivity between adenoviruses and Hepatitis C virus (52). Most of these examples of heterologous immunity have demonstrated a role for cross-reactive T cells, but other mechanisms may also be contributing toward the overall effect. With the induction of cross-reactive T cells, besides enhanced or inhibited clearance of a virus, T cell immunodominance patterns may be altered resulting in unusual skewing of T cell repertoires (53). It has

been shown that heterologous virus challenge may lead to expansion of cross-reactive narrowly focused T cell repertoire and viral escape, whereas homologous viral challenge may allow expansion of more oligoclonal T cell responses (17, 54). It has also been suggested that memory T cells generated after an infection have a lower activation threshold and may be activated by the bystander effect local cytokines independent of TCR signaling, contributing significantly to heterologous immunity (55). Further, it must be highlighted that in most instances, heterologous immunity is not nearly as effective as specific immunity but may be sufficient to alter the otherwise severe course of a heterologous infection and mortality. The widespread overlap in heterologous immunity between these different viruses suggests that cross-reactivity among pathogens is prevalent.

A recent article demonstrated that sequential challenges of mice with Dengue virus (DENV), Yellow Fever virus (YFV), and Japanese Encephalitis virus (JEV), all members of flaviviruses, results in the induction of heterologous cellular and humoral immunity (56). Prior exposure to YFV and JEV produced high titer antibodies against DENV1, whereas prior exposure to DENV1 produced cross-reactive antibodies against JEV but not YFV (56). Interestingly, JEV and JFV primed mice demonstrated T cell cross-reactivity with each other, whereas DENV1 priming induced cross-reactive T cells against JEV but not YFV, paralleling the cross-reactivity demonstrated by antibodies. It was further demonstrated that humans also have cross-reactive T cells and antibodies similar to data obtained in mice. Overall, the results demonstrated that cross-reactive flavivirus immunity can provide enhanced protection to a heterologous infection.

There are also multiple examples of heterologous immunity between bacteria and viruses. Herpes virus infection in mice with MCMV and Murine c-Herpesvirus, has been demonstrated to induce protective immunity against bacterial pathogens such as *Listeria monocytogenes* and *Yersinia pestis* (57). It has been suggested that increased levels of IFN-γ induces activation in macrophages resulting in enhanced clearance of intracellular bacteria. Besides epidemiological studies suggesting induction of heterologous immunity upon BCG immunization as described in earlier section, BCG has also been shown to provide protective immunity to VV in mice, and this protection appears to be dependent on cross-reactive $CD4^+$ T-cells (38). In contrast, BCG does not provide cross-reactive immunity against LCMV or MCMV in mice (38). A number of other heterologous effects of BCG appear to be related to its effect on innate immunity (58) and are not described here.

It has been demonstrated that a fungal species *Candida albicans* hyphal wall protein (Hyr1p) shares significant structural homology to a bacteria species *Acinetobacter baumannii* cell surface protein, and active (with rHyr1p) or passive (with anti-Hyr1p antibodies) immunization of mice protects them from systemic infection with *A. baumannii* and pneumonia (59). The observed cross-reactive/heterologous immunity among fungal and bacterial antigens was likely due to highly conserved B cell epitopes and 3-D structural homology between them. Most experimental studies of heterologous immunity have used animals (mice) immunized or challenged with a pathogen followed by determining the immune response or protection against an unrelated organism.

Heterologous immunity is rather difficult to demonstrate in humans due to continuous exposure with a number of pathogens, in comparison to inbred mice raised in a controlled laboratory environment. Furthermore, due to constant exposure to various pathogens, the memory T cell pool of an individual is also constantly changing. In an adult human, cross-reactive T cells represent a pool of cells ready to respond to a new pathogen. The quality and quantity of these cells are ultimately dependent upon an individual's immune history resulting from previous infections. T cell responses to a defined Hepatitis C virus (HCV) encoded HLA-A2-restricted non-structural protein 3 derived epitope NS31073–1081 was found to stimulate a cross-reactive T cell response to an Influenza virus encoded Neuraminidase antigen derived NA231–239 epitope in HCV-naive individuals (60–62). Similarly, our studies have demonstrated T cell responses against a number of HCV antigens in individuals who are otherwise HCV-naïve but are seropositive for Adenovirus (52). An earlier study also reported an abundance of pre-existing memory T cells against HCV NS3-1073 epitope from healthy HLA.A2 positive HCV-seronegative donors. Low dose exposure or acute clearance of HCV of the cohort was excluded in this study, and their origin from previous heterologous infections was suggested (63). Cross-reactivity of $CD8^+$ T cells generated against influenza antigen with HCV NS3-1073 epitope was also demonstrated to result in severe liver pathology in 2 out of 8 acute HCV-infected patients (64). Broad cross-reactivities in T cell responses have been demonstrated between Epstein-Barr Virus (EBV) and Influenza virus epitopes (65). Further, despite broad cross-reactivities, it has been shown that selective $CD8^+$ cross-reactive T cell repertoires against M1 antigen of influenza A virus and the early antigen BM of EBV play a significant role in disease severity of acute infectious mononucleosis during the acute EBV infection (66).

Infection with Dengue virus (DENV) in humans can sometimes lead to dengue hemorrhagic fever and shock syndrome. This severe immunopathology following DENV infection has been associated with re-exposure of individuals immune to one strain (serotype) of DENV with another strain. It has been demonstrated that cross-reactive non-neutralizing antibody can bind to viruses without inactivating them and enhance the infection of macrophages that bear Fc receptors for those antibodies (67). Furthermore, extensive T cell cross-reactivity occurs between different serotypes of DENV and a T cell response to the second dengue virus infection may induce CD8 T cells that have a higher affinity to the previously encountered Dengue virus, dampening the T cell response to the second virus. Therefore, both antibody-dependent immune enhancement and cross-reactive, low affinity T cell responses may combine to exacerbate the disease pathology (68).

NATURAL RESISTANCE TO INFECTIONS IN THE FRAMEWORK OF HETEROLOGOUS IMMUNITY

Infection of humans with various pathogens such as Influenza virus, Respiratory Syncytial Virus (RSV), HIV, Hepatitis B Virus (HBV), HCV, DENV, Zika Virus (ZikaV), West Nile Virus (WNV), Poliovirus, and EBV leads to variable outcomes with respect to self-clearance, severe pathology, mortality and/or persistence of infection (69). Humans are not naïve to foreign antigens and pathogens. Previous exposure to pathogens leads to induction of innate and adaptive immune responses, which result in establishing a substantial pool of memory B and T cells long after the pathogen insult has been eliminated. These memory cells provide a fast and efficient protective response upon re-exposure to the same pathogen/antigen, fulfilling the specificity, and memory mandates of adaptive immunity (**Figure 2**). However, these also form a pool of ready-to-respond cross-reactive cells with low stimulation requirements. Demonstration of T cells reactive against various antigens of viruses such as HCV, HIV, Human Cytomegalovirus (HCMV), and herpesviruses, from individuals never-exposed or naïve to these pathogens, point to the existence of cross-reactive T cells (70–72). However, their precise contribution to success

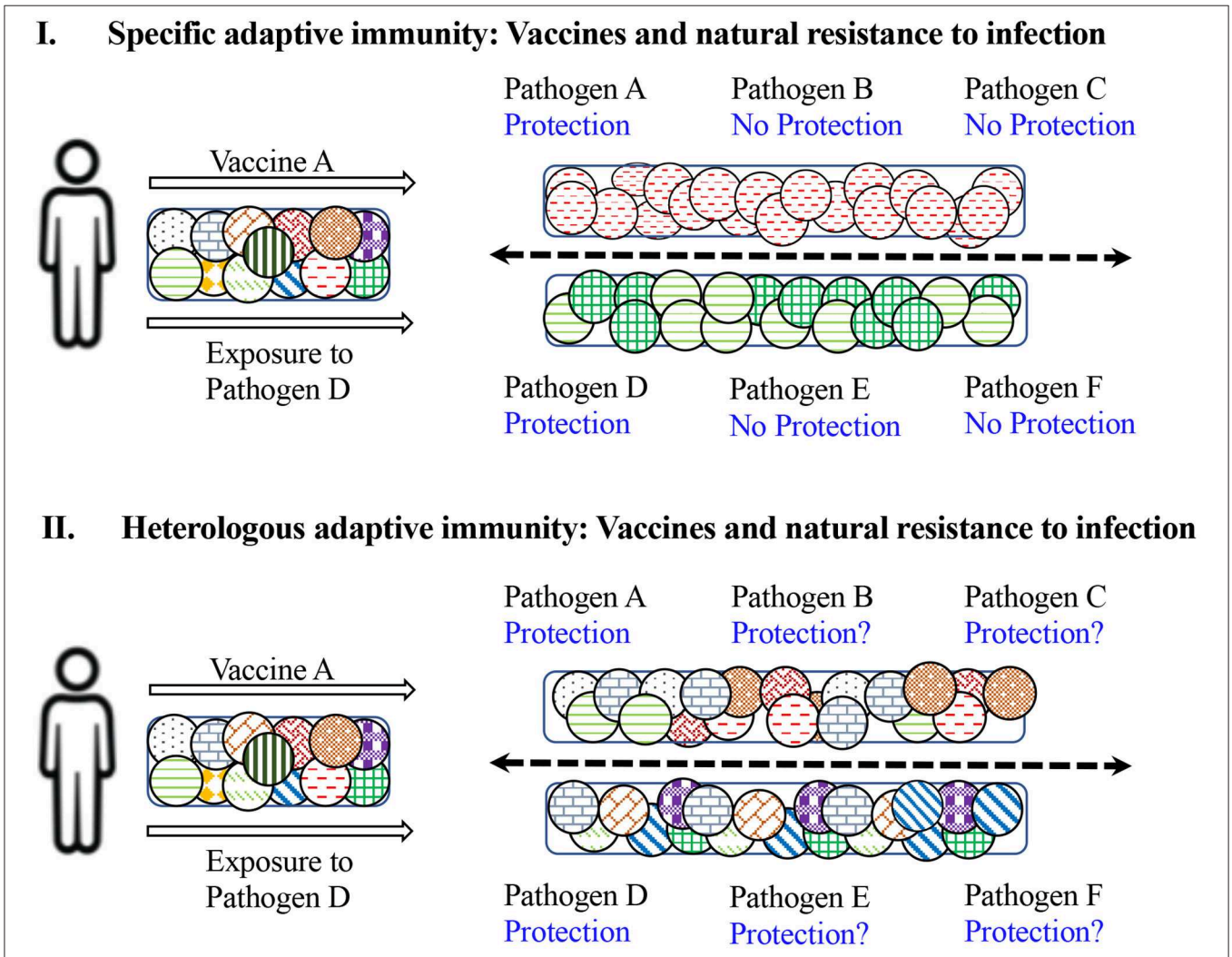

FIGURE 2 | Impact of specific vs. heterologous adaptive immunity on natural and vaccine-induced resistance to infection. **(I)** Depicts the results of specific immunity. Upon administration with vaccine against pathogen A, a limited repertoire of naïve T cells specific to antigens of vaccine A will be induced and an individual would be protected against subsequent exposure with pathogen A, but not against pathogens B and C. Similarly, exposure or infection with pathogen D will stimulate and expand T cells specific against D and protect against re-infection with pathogen D but not against subsequent infections with pathogens E and F. **(II)** Demonstrates the consequences of heterologous immunity. Upon administration with vaccine against A, a broad repertoire of cross-reactive T cells will be activated and expanded, and an individual would be protected against subsequent exposure with pathogen A, but also protected against pathogens B and C to some extent. Similarly, exposure or infection with pathogen D will induce cross-reactive T cells and protect against re-infection with pathogen D, but may also protect against subsequent infection with pathogens E and F. Therefore, the exposure history of an individual may modulate the outcome of future infections with multiple pathogens.

or failure of infection with these pathogens is still not clear (**Figure 2**).

As an example, infection with HCV presents an interesting scenario. There are currently ∼70 million chronically infected patients worldwide (64) (https://www.who.int/news-room/fact-sheets/detail/hepatitis-c). In 70–85% of the exposed individuals, chronic persistent infection follows, whereas the remaining 15–30% successfully clear asymptomatic or symptomatic acute infection. Induction of immune responses has been shown to be delayed in infected individuals, but it is not clear if some pre-existing cross-reactive immunity plays a role in spontaneous clearance of an acute infection (73). An interesting clinical observation has been that super-infection with Hepatitis A Virus (HAV), Hepatitis B virus (HBV), and Hepatitis D virus (HDV) in HCV-positive patients is associated with decreased HCV-RNA replication or HCV clearance (74). Further, three lines of observations suggest the role of cross-reactive immunity in natural immunity/resistance to HCV: (1). T cells against various HCV antigens are found in the blood of individuals who are not HCV-positive, not at risk for being HCV-positive and are seronegative for HCV (62, 63, 70, 75), and our earlier studies showed induction of TH1 or TH2 types of T cell responses from HCV-naïve individuals in an ∼1:2 ratio, which may reflect their propensity to develop acute vs. chronic infection upon exposure (76). (2). In seronegative individuals, HCV clearance has been reported after acute infection (60). (3). In some patients with persistent infection, spontaneous resolution of infection and seroconversion to antibody negative status has been found (77). In a group of blood donors with negative or indeterminate presence of anti-HCV antibodies and negative HCV RNA levels, a significant proportion had T cells that showed HCV antigen-specific T cell responses (78). Possible factors to explain this include (a) prior undetectable HCV exposure followed by clearance resulting in the induction of T cells reactive against HCV antigens, (b) abortive infection, or (c) occult infection with HCV. However, the possibility of cross-reactive T cells showing such responses cannot be excluded, especially since our studies have demonstrated robust cross-reactivity between commonly found adenoviruses (Ad5) and HCV (52, 79). The intriguing example of HCV demonstrates the complexity in determining the role of heterologous immunity in pathogenesis or protection from an infection, but undoubtedly indicates its significant role in natural immunity against a pathogen.

ROLE OF MICROBIOTA IN HETEROLOGOUS IMMUNITY

In addition to infectious pathogens, a host harbors a rich ecosystem of microbes in the order of 100 trillion microbes/person, consisting of Archaea, viruses, bacteria, fungi, protozoa and helminths. These microbiota are enriched at mucosal barriers such as gut and respiratory mucosa. Microbiota play an integral role in immune and metabolic homeostasis in an individual and can also contribute to control or prevent infection with a pathogen (80). Microbiota can either directly inhibit infection with pathogens by competing for an available niche, or indirectly inhibit infection through immune-mediated mechanisms (81). The microbiota can train/activate the innate immune system including antigen presenting cells, NK cells and innate lymphoid cells (ILCs), as well as induce a cross-reactive B and T cell repertoire able to recognize pathogens. Microbiota can also either allow the persistence of peripheral memory T cells or induction of regulatory T cells to induce tolerance to some pathogenic antigens. It has been suggested that the variety of antigens derived from the members of the microbiota can prime and lead to a diverse repertoire of memory T and B cells. These, in turn, can demonstrate enhanced immunity to a newly exposed pathogen through cross-reactivity (81).

Humans do not express the carbohydrate Galα1-3Galβ1-4GlcNAc-R (α-gal) and antibodies (both IgG and IgM) directed against α-gal are prevalent in human blood. Induction of α-gal-specific Abs is thought to be driven by exposure to bacterial components of the microbiota that express α-gal (82), such as *Klebsiella* spp., *Serratia* spp., and strains of *Escherichia coli*. Antibodies against α-gal have been shown to protect humans against the transmission of malaria (a protozoan *Plasmodium* infection), demonstrating the role of gut microbiota-induced specific acquired immunity that provides heterologous protection (83). Antibodies against α-gal have also been shown to be produced in humans infected with Gram-negative bacteria *Salmonella* spp. and protozoan parasites *Trypanosoma* spp. (84). It remains to be examined whether commensal- induced anti-α-gal antibodies also mediate heterologous protection against *Salmonella* spp. and *Trypanosoma* spp.

Microbiota-mediated induction of pre-existing antibodies against HIV gp-41 has been shown to be detrimental to the induction of neutralizing antibodies against HIV Env gp-120. This was seen in recipients of a DNA primed–rAd5 boosted HIV-1 vaccine in clinical trials (85) where a microbiota-mediated heterologous immune responses had a negative effect on vaccine efficacy.

VACCINE VECTORS IN THE CONTEXT OF HETEROLOGOUS IMMUNITY

The most successful human vaccines so far have been live attenuated viral or bacterial pathogens such as Measles, BCG, Poliovirus (oral polio vaccine), and Smallpox virus. These vaccines tend to induce life-long immunity. The successes of these vaccines led to their application as delivery vectors for an unrelated antigen in their makeup. Subsequently, various attenuated bacteria such as *Escherichia coli*, *Vibrio cholerae*, lactic acid bacteria (LAB), BCG, *Listeria* spp., *Shigella* spp., *Salmonella* spp., and viruses such as Pox viruses, measles, modified vaccinia Ankara (MVA) and replication-deficient adenoviruses (Ad) have been tested for the targeted delivery of various antigens of bacterial, viral and parasitic origin into a variety of animal hosts (86).

Ad are commonly used as vectors to deliver transgenes in gene therapy and vaccines (87–89). Natural exposure to Ad

is prevalent in the human population (up to 90% in some parts of the world based on antibody detection) and may lead to induction of neutralizing antibodies, which may reduce the generation of immunity against the transgene antigen (89). In addition to respiratory exposure, Ad have been shown to remain in the human gut for extended periods, making them akin to members of gut microbiota (90). Neutralizing antibodies against the vaccine vector has been the most studied response with respect to the immune response to Ad vectors, however it has also been suggested that by modifying the subtype/serotype, route and dose of Ad vector vaccines, one can circumvent the detrimental effect of pre-existing neutralizing antibodies. Consequently, rare human Ad and Ad of different animal species such as from cattle and chimpanzees have also been tested as vaccine vectors (91, 92). Although it has been shown that neutralizing antibodies don't cross-recognize Ad of different serotype or species, T cell cross-reactivity between different Ad has been demonstrated in humans (93). It has been shown that in contrast to B cell epitopes, common T cell epitopes are present in conserved regions (∼80%) of the Ad hexon protein. Due to the presence of conserved T cell epitopes in hexons, cross-reactivity among divergent serotypes from chimpanzees and humans has been observed. It has been shown that Ad-specific T cells are universal in humans even in low prevalence areas of the world, although the magnitude of $CD4^+$ and $CD8^+$ T cell response may vary among individuals. Further, it has been suggested that Ad-specific $CD8^+$ T cells remain in an effector-memory-like state and can readily and rapidly perform effector functions upon re-stimulation. By virtue of T cells being cross-reactive, these pools of Ad-specific T cells that are present universally in humans may provide an efficient source of effector T cells to target heterologous pathogens that regularly infect people.

Our studies have demonstrated unexpected and surprising homologies between peptides of HCV antigens and Ad antigens, and robust cross-reactive cellular and humoral immunity between Ad and HCV (52). It remains to be investigated whether Ad-specific effector-memory T cells provide the immediate defense when exposed to infecting HCV, and whether they form at least a partial basis for the 15–30% spontaneous clearance of HCV observed in humans. In a follow up report, we demonstrated that cross-reactive cellular and humoral immune responses against HCV antigens core, NS3, and NS5 are also induced upon immunization with various recombinant Ads containing antigens from HCV, *Mycobacterium tuberculosis*, HIV, and EBOV (94). Intriguingly, the nature of the transgene antigen had a significant impact on the levels of cross-reactive immunity induced against HCV antigens (94). It is possible that Ad also has cross-reactivity with other pathogens, but this has not been explored yet. These observations shed light on another rather unstudied aspect of vaccine vectors and support the notion that heterologous immunity induced by vaccine vectors may lead to significant heterologous immunity against another pathogen possibly influencing the natural course of infection with that pathogen. Consequently, non-specific effects of vaccine vectors must also be examined thoroughly.

DISCUSSION AND FUTURE PROSPECTS

Specificity and memory are the two traits of the adaptive immune system exploited for the development and application of vaccines. It is expected that administration of a vaccine would induce vaccine-antigen-specific T and B cell memory responses, which upon exposure to the corresponding pathogen, would rapidly and specifically lead to prevention or clearance of the infection. Many prophylactic and therapeutic vaccines, experimental or clinical, include antigens in various forms from pathogens, tumors, allergens, and/or autoantigens, and are designed to employ specific adaptive immunity, supported by adjuvants which mobilize the collaboration of non-specific, innate immunity. Experimental and clinical evidence as well as theoretical constructs have clearly demonstrated that heterologous immunity or cross-reactivity of adaptive immune cells is not an isolated or accidental phenomenon, but rather a fundamental attribute of adaptive immunity, forming an integral part of the host defense system against pathogens under natural conditions. Application of heterologous immunity in vaccines can be contemplated in chronic infections and cancer, where antigen-specific lymphocytes have become anergic and/or exhausted and don't respond to a specific vaccine. Mobilizing cross-reactive adaptive immunity by a heterologous therapeutic vaccine may be an ultimate strategy to induce effective protective immunity in such chronic disease conditions. Furthermore, as stated earlier, cross-reactive lymphocytes may not be the only mechanism behind heterologous immunity, and trained innate immunity could explain some of the observations of heterologous immunity. Therefore, it should also be pursued in future vaccine design and efficacy studies.

In an individual, T and B cell repertoires originate by random VDJ gene rearrangement, however, their cross-reactivity and ability to respond to various antigens in a host is shaped to a large extent by exposure history and microbiota, and therefore, cross-reactivity remains largely unpredictable at the individual level. Acknowledgment of the heterologous side of adaptive immunity does have important consequences on self-tolerance, autoimmunity, and vaccination strategies. Potential positive heterologous effects of vaccines have been discussed in this article, but heterologous immunity can also have dire consequences upon cross-reacting to self-antigens. It is essential that future investigation of vaccine design must exploit the beneficial aspects of heterologous immunity and at the same time devise strategies to avoid the potentially harmful effects. As stated earlier, cross-reactivity may vary among individuals and different sexes, and may largely remain unpredictable making application to vaccines which are usually population-based, not an easy task. In addition, recent understanding of the role of microbiota in immune homeostasis and induction of immune tolerance in a host has opened new avenues of investigation in vaccines. Specifically, it has been shown that pathological precipitation of many of the autoimmune diseases e.g., multiple sclerosis, type 1 diabetes, rheumatoid arthritis and systemic lupus erythematosus, are controlled to a large extent by environment and microbiota (95, 96). Moreover, even in individuals with genetic susceptibility and the peripheral presence of potentially

autoreactive T cells, modifications of gut microbiota may allow modulating the disease pathology (97). Therefore, it can be inferred that self-reactive (autoimmune) consequences of vaccines may be prevented/circumvented through modulation of gut microbiota. Additionally, development of tools and techniques to predict cross-reactivity against both unrelated pathogens and self-antigens would aid in vaccine design and coverage of protection obtained by a vaccine.

AUTHOR CONTRIBUTIONS

BA collected the literature, wrote, and revised the manuscript.

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Conflict of Interest: BA is coinventor on a PCT application relating to heterologous immunity between adenoviruses and Hepatitis C virus.

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BCG-Induced Cross-Protection and Development of Trained Immunity: Implication for Vaccine Design

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The Bacillus Calmette-Guérin (BCG) is a live attenuated tuberculosis vaccine that has the ability to induce non-specific cross-protection against pathogens that might be unrelated to the target disease. Vaccination with BCG reduces mortality in newborns and induces an improved innate immune response against microorganisms other than *Mycobacterium tuberculosis*, such as *Candida albicans* and *Staphylococcus aureus*. Innate immune cells, including monocytes and natural killer (NK) cells, contribute to this non-specific immune protection in a way that is independent of memory T or B cells. This phenomenon associated with a memory-like response in innate immune cells is known as "trained immunity." Epigenetic reprogramming through histone modification in the regulatory elements of particular genes has been reported as one of the mechanisms associated with the induction of trained immunity in both, humans and mice. Indeed, it has been shown that BCG vaccination induces changes in the methylation pattern of histones associated with specific genes in circulating monocytes leading to a "trained" state. Importantly, these modifications can lead to the expression and/or repression of genes that are related to increased protection against secondary infections after vaccination, with improved pathogen recognition and faster inflammatory responses. In this review, we discuss BCG-induced cross-protection and acquisition of trained immunity and potential heterologous effects of recombinant BCG vaccines.

Keywords: BCG, innate immunity, trained immunity, heterologous protection, vaccine

INTRODUCTION

One of the leading causes of human death worldwide is tuberculosis (TB), a bacterial infection caused by *Mycobacterium (M.) tuberculosis*. In 2017, 10 million people developed TB disease, causing 1.3 million deaths (1). To prevent TB, a vaccine was developed in 1921 by Albert Calmette and Camille Guérin, which is currently included in the immunization programs of most countries.

This vaccine consists of an attenuated *M. bovis* bacillus that was repeatedly passaged in culture by Calmette and Guérin and which is known as the bacillus Calmette-Guérin (BCG) (2). This vaccine was developed from a virulent *M. bovis* strain that accumulated more than 14 genome deletions in different regions (3). In most countries, BCG is administered to newborns a few hours or days after birth and its protective effects against tuberculous meningitis and miliary tuberculosis (TB) have shown efficacy over 70%, while its protective effect against pulmonary TB displays an average of 52% of protection (4–7). Mathematical estimations suggest that BCG vaccination of 100.5 million children, out of the 132.8 million children born in the world, prevented nearly 30,000 cases of TB meningitis and 11,500 cases of miliary TB in the year 2002 (6). In adults, BCG vaccination fails to completely protect against pulmonary TB, showing a range of effectiveness between 0 and 80% (8–10), which explains why TB is one of the major causes of mortality worldwide (1). Despite this, in 2018 BCG was considered within the national vaccination program of 154 countries, including countries in America, Asia, Africa, and Europe, with coverage of over 90% (1). It was also administered to high-risk groups in additional countries, being one of the most widely used vaccines worldwide (1, 11). Besides protecting against TB, BCG vaccination also reduces mortality in children because of non-specific cross-protection induced by this vaccine against other unrelated pathogens (12, 13). Initial evidence for this phenomenon was described in Sweden in 1927 by the physician Carl Näslund, who found that during the first year of life, BCG-vaccinated newborns had a mortality rate that was three times lower than unvaccinated babies (14). This observation was also made by Albert Calmette, in 1931 (15). In Guinea-Bissau, a country with a high childhood mortality rate, the presence of a BCG-vaccination scar was associated with diminished mortality rates associated with malaria or unclassified fever (16). Besides, BCG-vaccinated children showed a reduced risk of developing acute lower respiratory tract infections (ALRI) as compared with non-vaccinated ones (17). Furthermore, several studies carried out in West Africa showed over a 40% reduction in mortality after BCG vaccination, preventing malaria, sepsis, respiratory infections, and leprosy (14, 16, 18–21). In Spain, BCG vaccination reduced hospitalizations due to respiratory infections unrelated to TB in children under 14 years of age (13). Also, reduced child mortality due to BCG vaccination has been observed in other places of the world, including Sweden, United Kingdom, South or Southeast Asia, India, and Haiti (22–24).

Another remarkable characteristic of BCG is that it can be used as an expression vector for recombinant antigens to develop novel vaccines for pathogenic bacteria and viruses (25–34), as well as for cancer diseases (35–43). BCG has been considered as a good vector given its safety shown in vaccinated neonates, children and adults for almost a 100 years and that BCG antigens may act as adjuvants, inducing innate and adaptive immune responses (11, 22–24, 44, 45).

IMMUNE RESPONSE INDUCED BY BCG VACCINATION

The immune response elicited after BCG vaccination begins at the inoculation site after intradermal injection, where resident neutrophils, macrophages, and dendritic cells (DCs) interact with the bacillus (44, 46). The recognition of BCG by immune cells takes place through the interaction of different pattern recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs), such as peptidoglycan, arabinogalactan, and mycolic acids located at the bacterium cell wall (44). Among the receptors involved in the recognition of BCG are toll-like receptors (TLRs) TLR2 and TLR4 present on the cell surface membrane (44). It has been shown that different proteins expressed by *mycobacteria* can work as TLR agonists, stimulating macrophage, and DC maturation and the secretion of pro-inflammatory cytokines (47). Likewise, complement receptors CR3 and CR4 are involved in the recognition of opsonized *mycobacteria* by DCs. Another group of cell receptors that recognize BCG PAMPs are nucleotide-binding oligomerization domain (NOD)-like receptors found in the cytosol of innate immune cells, such as NOD2, which interact with a specific component of the bacterial peptidoglycan (48). Besides, C-type lectins, such as DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) interact with components of the bacterial wall and are involved in the recognition and internalization of BCG (48). After internalization by DCs, the mycobacterium can live up to 2 weeks inside these cells (49). This interaction induces DC maturation and migration that is characterized by an increase in the expression of co-stimulatory molecules, such as CD40, CD80, CD83, and CD86 (50). One of the antigens present in the cell wall of BCG corresponds to antigen (Ag) 85 (also present in *M. tuberculosis*), which stimulates the production of tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β) and IL-6 (51, 52), which are able to generate an pro-inflammatory state that promotes the activation of immune cells (50).

The development of an adaptive immune response starts when antigen-presenting cells (APCs, e.g., DCs, macrophages and B cells) present antigenic peptides on MHC molecules and prime T cells located at the nearest secondary lymphoid tissues or the spleen (53). *In vitro* and *in vivo* studies have shown that BCG-infected skin DCs migrate to the draining lymph nodes where they secrete TNF-α, IL-6, and IL-12 and activate both, $CD4^+$ and $CD8^+$ T cells (54–57) (**Figure 1**). Interestingly, it has been reported that BCG-infected human neutrophils cooperate with infected DCs to stimulate antigen-specific T cell responses (58).

As summarized in **Figure 1**, the adaptive immune response induced after BCG vaccination involves the activation of both, $CD4^+$ and $CD8^+$ T cells (53, 59) with elevated production of IFN-γ, which increases the anti-mycobacterial activity of macrophages (52, 53). This cytokine also contributes to the activation of B cells and the subsequent generation of antigen-specific antibodies by plasma cells. In early stages after vaccination, a pool of mycobacteria-specific $CD8^+$ T cells

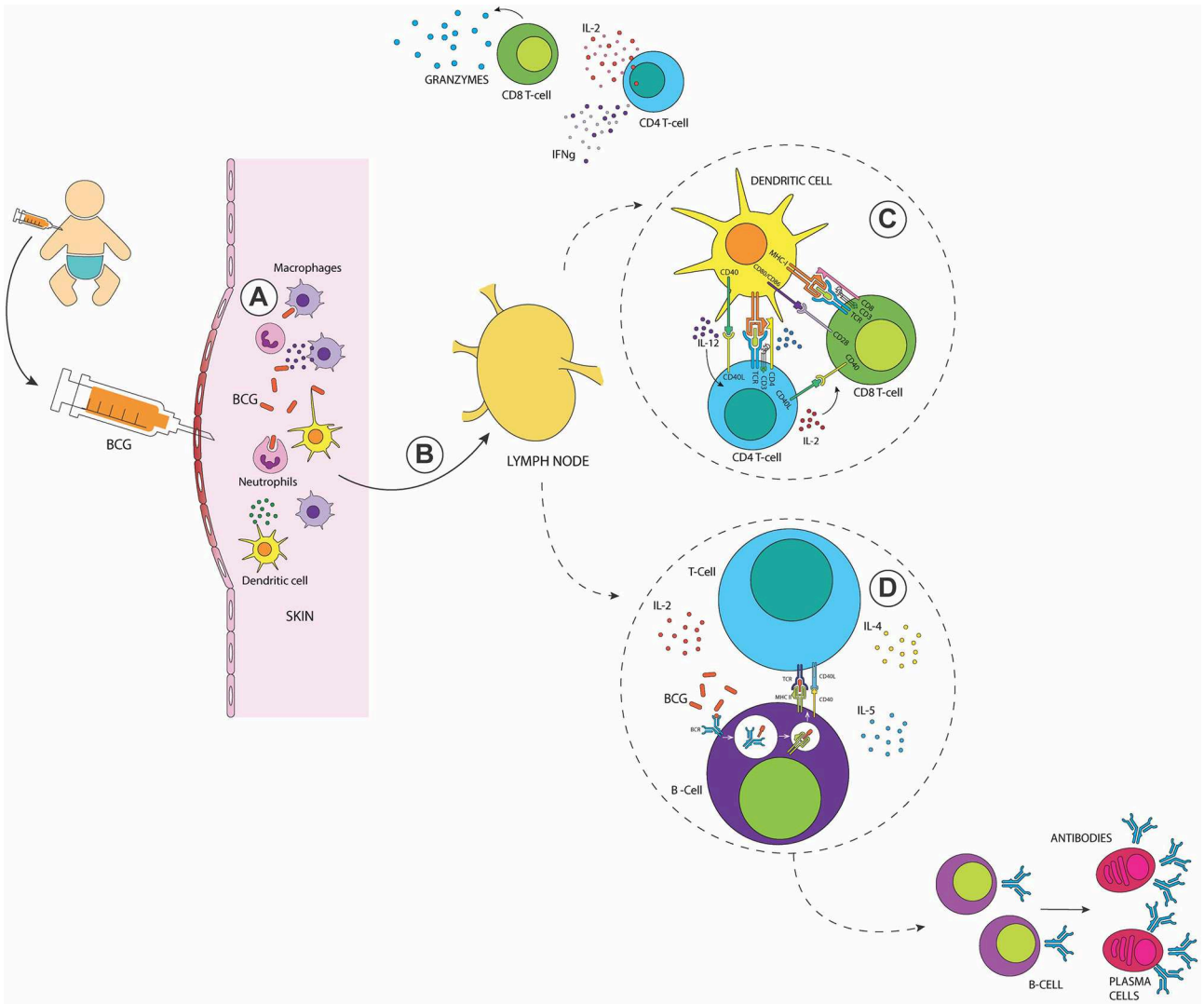

FIGURE 1 | The immune response elicited after BCG vaccination in the newborn. **(A)** Recognition of the BCG at the inoculation site by neutrophils, macrophages, and DCs. **(B)** Activated skin DCs migrate to the draining lymph nodes to activate adaptive immune cells **(C)** Activation of Mycobacteria-specific $CD4^+$ and $CD8^+$ T cells with a T_{H1} profile, secreting elevated amounts of IFN-γ and granzymes **(D)** Activation of B cells leads to the generation of memory and plasma cells and the production of antigen-specific antibodies in response to the presence of antigens of BCG. After their activation, memory T and B cells reside in lymph nodes.

proliferates and is present in peripheral blood up to 10 weeks after BCG vaccination (60). These $CD8^+$ T cells were able to secrete IFN-γ and express granzymes, as well as perforins supporting the cytotoxic potential for these cells (60, 61). Activated T_{H1} $CD4^+$ T cells have also been detected (62, 63), which produce large amounts of IFN-γ, TNF-α, and IL-2 (55, 64). In newborns, BCG-specific $CD4^+$ T cells could be detected in the peripheral blood 3 weeks after vaccination, with a peak at 10 weeks (60). Studies with T cells transferred from BCG-vaccinated mice into animals that are deficient for both B and T cells have shown that $CD4^+/CD8^+$ T cells are necessary for reducing and controlling bacterial dissemination (65). During the contraction phase, BCG-specific $CD4^+$ and $CD8^+$ T cells switch to a memory phenotype, with functional features of effector memory T cells secreting IFN-γ (64, 66). These memory T cells generate a strong lymphoproliferative response to TB antigens several months after vaccination in mice (66).

Between 4 and 8 weeks after BCG vaccination, there is an induction of a B cell response that increases the production of IgG (67) and induces long-lived memory B cells (68). These IgG molecules can opsonize BCG and *M. tuberculosis*, enhancing phagocytosis and the inhibition of intracellular bacterium growth (67). It was shown that mucosal BCG vaccination induces an airway-resident memory T cell population in the lungs, but immunoglobulin production was not measured in this study (69). Pulmonary immune response in mice infected with *M. tuberculosis* was improved when BCG was administered intranasally as compared to subcutaneous vaccination (70).

This improved protective efficacy was concordant with an increased presence of IgA and $CD4^+IL\text{-}17A^+$ T cells in bronchoalveolar lavages of intranasally vaccinated mice (70). Although subcutaneous BCG vaccination enhances blood IgG levels, in the case of respiratory airway pathogens, IgA confers better protection against infections (70). This is due to the fact that IgA is constitutively found in serum and mucosa and corresponds to one of the first barriers of defense (71). These antibodies can neutralize, excrete pathogens and activate the immune response by the modulation of the secretion of cytokines, such as TNF-α and IL-1β (71).

BCG VACCINE AS A STRATEGY FOR MODULATING IMMUNITY

Besides protection against TB, BCG has other clinical applications, especially in two main immunotherapy fields: treatments for cancer and autoimmune diseases, such as melanoma and type-1 diabetes (T1D), respectively (we summarize the contribution of BCG to immunotherapy in **Figure 2**). T1D is an autoimmune disease characterized by the destruction of pancreatic beta cells (72). This destruction leads to a lack of insulin production, which leads to the development of hyperglycemia, polyuria, and hypoinsulinemia (73). The effect of BCG vaccination in T1D is still controversial, as it was originally observed that BCG vaccination promoted a remission of the disease when patients were treated during the first month after diagnosis (74). On the other hand, a randomized clinical trial performed in 1999 where patients between 5 and 18 years old were vaccinated early after disease appearance showed no difference in glycated hemoglobin levels (HbA1c, an indicator of blood glucose levels) or endogenous insulin secretion compared to non-vaccinated ones (75). In a phase I trial performed in adults with long-term T1D, BCG vaccination in multiple doses was able to reduce HbA1c levels and increased the death of insulin-autoreactive T cells (76). Interestingly, BCG modulation of blood sugar was associated with a systemic shift toward a glycolytic pathway of glucose utilization (76). Another clinical trial performed with long-term T1D patients showed that vaccination with BCG stabilized HbA1c levels without producing hypoglycemia, an effect that could last up to 8 years after vaccination (77). In non-obese diabetic (NOD) mice, injection of BCG was shown to reduce insulitis and diabetes development (78). It has been demonstrated that the stimulation of TNF-α induced by BCG is involved in the destruction of insulin-autoreactive T cells (79). Despite this, the mechanism involved in the immunomodulatory effect induced by BCG in T1D patients has not yet been elucidated.

Another important autoimmune disease is multiple sclerosis (MS), which is characterized by the development of neurological symptoms due to gradual demyelination of the central nervous system (CNS) as a consequence of an inflammatory-autoimmune response (80). At present, a specific treatment for this disease that is effective is not available. Nonetheless, clinical trials showed that BCG vaccination could reduce the frequency of active lesions in the CNS of 12 MS patients (81). Further, a phase II clinical trial showed that after the first demyelinating episode, BCG vaccination reduced the risk of developing clinically definite MS for 5 years (82). Moreover, in a widely used mouse model of MS named experimental autoimmune encephalomyelitis (EAE), the injection of subcutaneous extended freeze-dried (EFD) BCG attenuated the severity of EAE (83). Mice treated with EFD BCG showed significantly lower clinical scores and reduced infiltration of $CD45^+$ cells in the spinal cords (83). Furthermore, this treatment was shown to reduce the frequency of T_{H17} cells and to increase the frequency of T_{REG} cells in secondary lymph nodes. This consequence will help to limit the inflammation induced by EFD BCG in EAE (83).

Since several studies have demonstrated that BCG induces T_{H1}/T_{H17} responses against TB and other unrelated pathogens (62, 84, 85), its capacity to exert a regulatory effect over autoimmune diseases, such as T1D and MS is very surprising. However, there are immune-metabolic pathways involved in the activation of the immune system after BCG vaccination that may account for explanations of these observations. Indeed, the activation of innate immune cells and T cells induced by this vaccine is partly mediated by the activation of cell glycolytic pathways (86). Besides, human Treg cells are highly glycolytic (87). Based on these findings, Ristori et al. proposed in 2018 that BCG induces a tolerogenic response via enhancement of glycolysis, contributing to the reduction of inflammation in autoimmune diseases (88). Another possible mechanism through which BCG can mediate protection in the context of autoimmune diseases relays on the immune response to the infection with the mycobacterium. After infection, it has been shown that activated, but not naïve, $CD4^+$ T cells undergo apoptosis in an IFN-γ-dependent manner (89). Thus, apoptosis of activated T cells may have as a consequence the diminution of activated autoreactive cells, improving the health condition of the individual receiving vaccination. Also, TLR-signaling stimulated by mycobacterial components induces IL-10 secretion by B cells and consequent suppression of Th1 and Th17 activities, contributing to the suppression of autoimmune reactions (90).

On the other hand, there is increasing evidence for the use of BCG vaccination for the prevention and treatment of cancer (35–43). Vaccination of newborns with BCG reduces the risk of developing melanoma (35) and childhood leukemia (36). In the case of melanoma, direct vaccination with BCG into nodules of intradermal or subcutaneous metastases induced their regression in 90% of the injected lesions (37). As previously described, BCG exposure of tumoral macrophages induces transcriptional reprogramming of these cells, leading to an improved pro-inflammatory phenotype (91). BCG-treated macrophages can induce the activation of T cells infiltrating the tumor, thus improving antitumor immunity (91). Moreover, in patients with non-muscle invasive bladder cancer, it has been shown that BCG instillation after transurethral resection reduces tumor progression (38, 92). BCG impairs the tolerogenic milieu developed by carcinogenic cells, inducing local infiltration of macrophages, $CD4^+$ T helper cells, $CD8^+$ T cells, and NK cells, resulting in the development of local inflammation (40, 41, 93). Bladder tumor cells express antigen-presenting and co-stimulatory molecules after being infected with BCG, suggesting

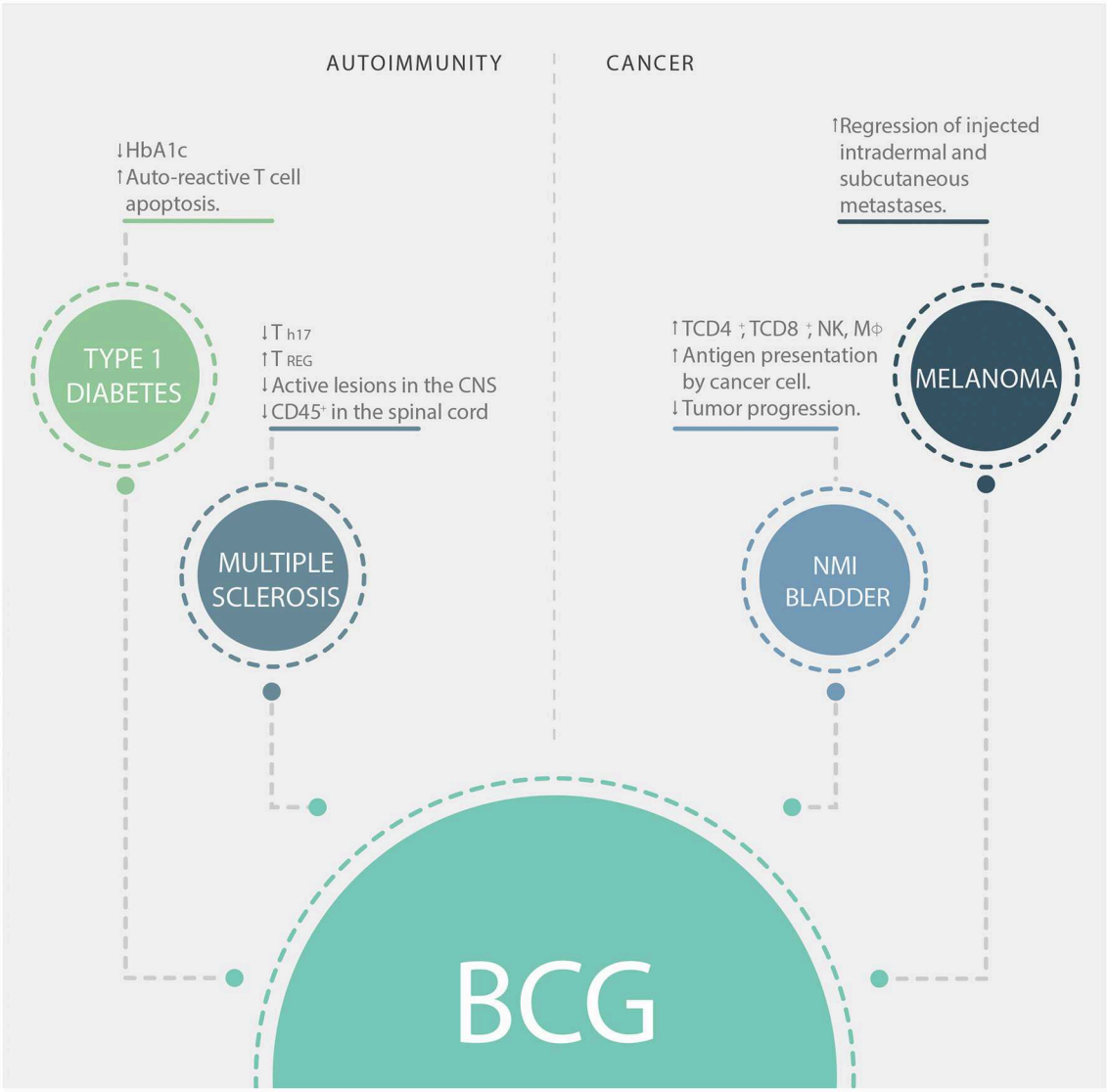

FIGURE 2 | BCG applications in immunotherapy. Clinical applications of BCG in autoimmunity (left boxes) and cancer (right boxes). HbA1c, glycosylated hemoglobin; CNS, central nervous system; NK, natural killer cells; Mϕ, macrophages; NMI, non-muscle invasive.

that these cells can function as antigen-presenting cells making them a good target to be destroyed by cytotoxic cells (42). The antitumor effects of BCG immunization could also be associated with the development of local inflammation that might overcome the tolerogenic environment induced by tumor cells (43).

The different effects observed after the administration of the BCG vaccine suggest that the activation of the immune system induced by it could vary depending on the environment in which it is found. BCG administration could exert a beneficial modulation of the immune system in the context of autoimmune diseases due to a redirection of the inflammatory response. In the case of cancer, the activation of the immune system in the presence of the bacteria within the tumor alters the tolerogenic environment induced by cancer cells, leading to a specific cytotoxic activity against the tumor cells.

TRAINED IMMUNITY AS A CONSEQUENCE OF BCG VACCINATION

Netea et al. (94) were the first to propose the concept of "trained immunity," which is defined as an increased non-specific response to a secondary infection mediated by the innate immune system, either to the same or different microorganisms (94). This type of immunity is characterized as being independent of T and B cell responses and is mediated by monocytes/macrophages and NK cells (95).

In humans, BCG vaccination of adults induces a trained phenotype in circulating monocytes, characterized by an increased capacity to produce proinflammatory cytokines, an effect that has translated to non-specific protection against unrelated pathogens, such as *S. aureus* and *C. albicans* (96, 97).

Also, BCG vaccination of healthy human volunteers increased the capacity of NK cells to secrete proinflammatory cytokines, such as IL-1β and IL-6 after stimulation with *M. tuberculosis* or unrelated pathogens (*S. aureus, C. albicans*) (98). These observations were performed 3 months after vaccination, consistent with the fact that BCG reduces mortality in newborns during the first year of life, as mentioned above. Thus, BCG induces non-specific protection against unrelated pathogens (96).

Interestingly, innate immune cells mediate this nonspecific protection, independent of T and B cells. A comparison of systemic lethal candidiasis infection in severe combined immunodeficiency (SCID) mice, which lack T and B cells, and NOD/SCID/IL2Rγ (NSG) mice that lack T, B and, NK cells, showed that partial protection was mediated by NK cells in BCG-vaccinated mice (98). Specifically, mice were challenged with a lethal intravenous dose of *C. albicans* 2 weeks after BCG vaccination; while SCID vaccinated mice survived, NSG vaccinated mice were partially protected, suggesting a role for NK cells in the non-specific protective effect induced by BCG vaccination (98).

Besides, there is *in vitro* and *in vivo* evidence of BCG-related trained immunity effects in bovine monocytes (99). *In vitro* exposure of calf monocytes to BCG leads to enhanced TNF-α and IL-6 production after subsequent TLR agonist stimulation. Aerosol BCG vaccination of calves exerted a similar effect of PBMCs, boosting pro-inflammatory cytokine production, in association with a shift to anaerobic glycolysis (99). The trained immunity phenotype was also observed testing PBMCs obtained 3 months after vaccination (99), which further supports previous studies carried out in human cells.

It has been shown that one of the molecular mechanisms that induces the development of trained immunity is epigenetic reprogramming, specifically through histone modifications (95). Epigenetic modifications regulate gene expression in response to environmental signals (100). In the immune system, epigenetic modifications are involved in cell differentiation, inflammation and autoimmune diseases (96, 100–103). Different types of epigenetic modifications have been described, including DNA modifications, non-coding RNAs, histone modifications and chromatin remodeling (100). Histone modifications are highly dynamic and can change within minutes; there are different classes of modifications such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization (104). Histones can be methylated at arginine or lysine residues. Lysine can accept up to three methyl groups, being mono-, di- or trimethylated. Arginine can be mono- or dimethylated (105). These modifications are involved in the activation or repression of the transcription of genes that they are associated with. While methylation of lysine 4 in histone 3 (H3K4), H3K36, and H3K79 are usually associated with transcription activation; methylation in H3K9, H3K27, and H4K20 are associated with gene silencing (105).

After BCG vaccination, peripheral blood monocytes show an increase in H3K4me3 histone modification associated with the promoters of the genes *tnfα, il6*, and *tlr4* that lead to the transcriptional activation of these proinflammatory cytokines (96, 106). These responses are dependent on the nucleotide-binding oligomerization domain 2 (NOD2) receptor present in monocytes and receptor-interacting protein kinase 2 (Rip2) (96). These epigenetic modifications upregulated the expression of pattern recognition receptors (PPRs), namely TLRs, C-type lectins receptors, NOD-like receptors, and RIG-I-helicases that specifically recognize pathogen-associated molecular patterns (PAMPs) and modulated the accessibility of transcription factors to proinflammatory cytokine genes (96). Consequently, when these trained monocytes are exposed to a second infection, the pathogen is recognized by PPRs, leading to increased cytokine production (95).

In addition to epigenetic reprogramming, different cellular metabolic pathways are involved in the regulation and development of trained immunity in monocytes, macrophages and NK cells (86, 107–109). Indeed, glycolysis metabolism is increased in human monocytes after BCG vaccination, leading to a shift in the metabolic programming of the cell from oxidative phosphorylation to aerobic glycolysis (Warburg effect) (108). Additionally, it has been demonstrated that inhibition of the glycolytic pathway impairs the development of a trained immunity phenotype by preventing epigenetic rearrangements (86). Specifically, it was shown that glycolysis inhibits epigenetic modifications at the promoters of genes encoding for IL-6 and TNF-α in peripheral monocytes (86). Glutaminolysis and cholesterol synthesis have also been involved in the development of trained immunity in monocytes, being fumarate a key metabolite that can induce chromatin rearrangements. This metabolite induces an increase in H3K4me3 in the promoters of *tnfa* and *il6* leading to elevated secretion of these cytokines upon re-stimulation with LPS (107). Additionally, accumulation of mevalonate, a metabolite of the cholesterol synthesis pathway, has also been shown to be able to induce trained immunity through the enrichment of H3K4me3 in the promoters of *tnfa* and *il6* (109). All these reports support the notion that epigenetic regulation is intimately related and coordinated with the metabolic state of the cell.

Furthermore, *all-trans* retinoic acid (ATRA) is a vitamin A metabolite involved in the development of tolerogenic immunity (110). This metabolite regulates tolerogenic cytokine production and cell differentiation in monocytes, macrophages, DCs and T cells (111). *In vitro* stimulation of BCG trained monocytes with ATRA inhibits H3K4me3 and induces a strong repressive hallmark (H3K9me) in the promoters of proinflammatory genes such as *tnfα, il6, il8, il10,* and *il1ra*. Consequently, the transcription of these genes is silenced, inhibiting the "trained" phenotype (111).

BCG-induced epigenetic reprogramming of monocytes was shown to be able to protect humans against an experimental yellow fever virus (YFV) challenge (112). BCG-vaccinated subjects showed lower viremia after infection with an attenuated YFV vaccine strain. Interestingly, trained immunity induced by BCG was modulated by IL-1β treatment *in vitro*, and cytokine production was increased after vaccination (112). These changes in cytokine secretion were mediated by an increase in H3K4me3 and reduction of H3K9me3 in the promoter regions of the genes *tnfα, il6,* and *il1β* (112).

The genetic reprogramming described above could be implicated in the generation of memory-like innate immune cells (94). In this context, after BCG vaccination, innate immune cells, such as monocytes would undergo a series of chromatin modifications (84, 86, 96). These chromatin rearrangements would lead to a "trained phenotype" that would generate an enhanced innate response when exposed to any non-specific pathogen (**Figure 3**). Interestingly, chromatin rearrangements induced by BCG vaccination can reprogram bone marrow progenitors, stimulating myelopoiesis, and generating trained immune cells with a higher capacity to protect against a wide variety of pathogens (113, 114). These characteristics of trained immunity suggest that innate immune cells could be a different target for vaccination. As reviewed by Khader et al., vaccination against *M. tuberculosis* with BCG could be directed to the generation of trained hematopoietic progenitors and, in combination with classic vaccination for adaptive immunity generation, generate a greater and more effective immune response (115).

RECOMBINANT BCG VACCINES

BCG has been considered a good expression vector for recombinant antigens due to several advantages. First, BCG administration is safe for neonates, infants, and adults. Second, BCG doses are relatively easy and non-expensive to produce, allowing for mass-production, and furthermore, it is temperature stable (45). Finally, BCG acts as auto-adjuvant and induces innate and adaptive immune responses (44). Several recombinant BCG (rBCG) strains that express heterologous antigens of different pathogens have been developed and tested since 1991 (25–34). These rBCGs formulations are excellent vaccine candidates, due to the auto-adjuvant characteristics conferred by BCG antigens.

In a mouse model of measles virus (MV) infection, rBCG expressing a nucleocapsid (N) protein of MV significantly reduced viral titers in brain homogenates and mortality due to measles-induced encephalitis (26). Splenocytes from vaccinated mice showed stronger proliferation of antigen-specific T cells in response to MV *in vitro* and an increase of serum MV-specific antibodies as compared to BCG-WT-vaccinated animals (26). This recombinant vaccine was later tested in infant rhesus macaques challenged with an intranasal inoculation of MV (116). Specifically, a total of eight newborn rhesus macaques received the rBCG-MV-N vaccine with no adverse effects, thereby demonstrating its safety. Moreover, rBCG-MV-N vaccination did not induce an increase in antigen-specific antibody titers or expansion of B cell follicles in lymph nodes. The determination of nasopharyngeal viral loads did not show significative differences in the vaccinated groups. Despite this, rBCG-MV-N vaccinated monkeys showed reduced lung pathology after viral challenge in comparison to those vaccinated with WT-BCG and paracortical hyperplasia in lymph nodes, suggesting that protection was mediated by specific T cells (116). Although vaccination with this rBCG was not able to prevent systemic infection, the reduction of lung pathology may prevent MV-associated deaths.

Toxoplasma gondii (*T. gondii*) is an intracellular protozoa parasite that infects a variety of warm-blooded animals and is one of the most prevalent human infections worldwide (117). Most immunocompetent humans do not develop clinical signs after acquired infection, but immunocompromised individuals are at risk of developing serious complications, including fatal ones (117). A rBCG expressing rhoptry protein 2 (ROP2), which is a protein of the *T. gondii* involved in host cell invasion induces antigen-specific immune responses in mice (27). Vaccination of mice with a rBCG expressing ROP2 induced the production of specific antibodies, which were detected in serum and induced cellular immune responses (27). Besides, delayed mortality was seen after infection with *T. gondii* in immunized mice (27).

Bordetella pertussis is a gram-negative coccobacillus that causes whooping cough (118). This disease causes serious complications including secondary bacterial pneumonia, apnea, bradycardia, pulmonary hypertension, and even death in infants younger than 6 months old (118). An rBCG expressing the S1 subunit of the *B. pertussis* toxin (dPT) was developed and tested as a vaccine in animals (119). Vaccination of mice showed that this vaccine was able to induce the secretion of antigen-specific antibodies and protect mice against a challenge with a lethal dose of B. *pertussis* (119–121). Stimulation of splenocytes of immunized mice with dPT antigen showed an increased capacity to secrete IFN-γ compared to the non-immunized group. Vaccination of 5-day old mice with the rBCG-S1PT vaccine protected them after a lethal dose challenge with *B. pertussis*, showing 100% survival, while non-vaccinated mice had 0% survival 8 days after challenge (121).

Human immunodeficiency virus (HIV) causes acquired immune deficiency syndrome (AIDS) in humans. According to the WHO, this syndrome caused 1.2 million deaths globally in 2016 (122). There is no effective vaccine preventing infection with this virus. Yet, there are many groups all over the world working on the development of an effective vaccine against this virus (28, 29, 31, 45, 123–127). The development of rBCGs expressing HIV antigens has been considered an interesting immunization strategy for a vaccine against HIV. An rBCG expressing the Env protein of the viral capsid of HIV has been shown to develop T_{H1} responses in mice (28). However, it was unable to induce the production of HIV-specific antibodies (28). An rBCG expressing viral antigens used in combination with a booster of viral vectors was also able to induce HIV-specific T cell responses in mice, which was characterized by the secretion of IFN-γ and a T_{H1} polarization (29). rBCG-HIVA, a recombinant BCG that expresses the H and P epitopes of the Env protein and viral polymerase respectively, has been shown to induce the activation of HIV-specific T cells in mice (30). This vaccine in combination with a recombinant viral vector induced robust T cell responses against HIV and *M. tuberculosis* (31). Although these are promising preclinical results, clinical trials must be done to evaluate their efficacy and protection in humans.

The human metapneumovirus (hMPV) is the second major cause of acute lower respiratory tract infections in children and the elderly. This viral infection induces inflammation and disruption of the lung architecture, causing bronchiolitis and pneumonia (128). There is no effective vaccine available to

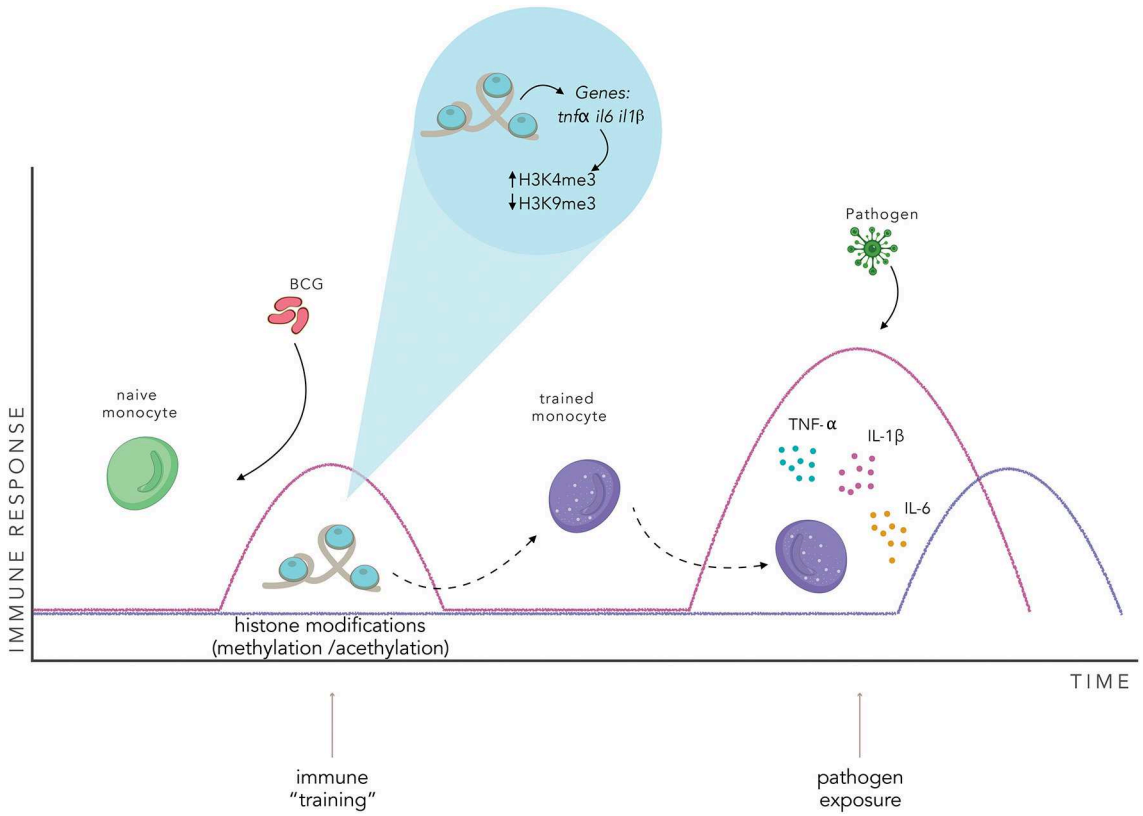

FIGURE 3 | BCG vaccination induces an innate immune training. BCG vaccination activates the innate immune system and induces changes in the pattern of histone modifications of specific genes in innate immune cells. This chromatin rearrangement induces a "trained" state in the cell, enhancing the effectiveness of the innate immune response when exposed to a non-specific pathogen, inducing the secretion of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6. The pink line represents a trained immune response, the purple line represents a naïve innate immune response.

prevent infection with hMPV. However, a novel vaccine has been developed with a rBCG expressing the phosphoprotein (P) protein of hMPV (rBCG-P-hMPV) (32). This rBCG-P-hMPV formulation induces a humoral response against hMPV and can induce viral neutralization and confer protection against hMPV-infection, lowering the amounts of viral particles in the lungs in vaccinated mice (32, 129). Vaccination with rBCG-P-hMPV also reduced T cell infiltration and tissue damage in a mouse model of infection with hMPV, activating a T_{H1}-type response and preventing the development of the disease (34).

Another relevant respiratory pathogen is the human orthopneumovirus (previously named human respiratory syncytial virus, hRSV), which is one of the leading causes of acute lower respiratory tract infections in the world (130). In 2015, hRSV caused over 33 million episodes of acute lower respiratory tract infections worldwide, being one of the major causes of hospitalization in children under 5 years of age (131). A rBCG vaccine has been developed expressing the nucleoprotein (N) of the hRSV (rBCG-N-hRSV) (32). In a mice preclinical model, the immunization with rBCG-N-hRSV confers protection against hRSV challenge, reducing both clinical pathology and neutrophil infiltration in the lungs (129). Noteworthy, this vaccine induces the secretion of viral-specific antibodies with neutralizing activity (32), which correlates with lower amounts of viral titers in the lungs in vaccinated mice (129). The rBCG-N-hRSV vaccine has been formulated under good manufacture practices (cGMP) and shown to maintain its promising results in pre-clinical trials. Immunization of mice with rBCG-N-hRSV induces a T_{H1}/T_{H17} memory response that was capable of mediating virus clearance and avoiding lung damage (129).

Even though these rBCG strains have shown promising results and protection against their target pathogens, no reports of cross-protection against unrelated pathogens have been published yet. Also, it remains unclear if they maintain the benefits of cross-protection granted by the wild-type strain. Nevertheless, it appears that the trained immunity induced by BCG vaccination could represent additional protection against the pathogen, whose antigen must be expressed by the rBCG. Interestingly, several reports have shown that there is some degree of protection induced by WT-BCG vaccination against other pathogens (95, 96, 106, 112, 132). In the case of *T. gondii*, the determination of IFN-γ secretion by splenocytes stimulated with recombinant ROP2 showed an increased capacity of cells from mice vaccinated with WT-BCG compared to non-vaccinated animals (27), suggesting non-specific protection by WT-BCG against toxoplasmosis. For *B. pertussis*, it is worth noting that splenocytes obtained from mice vaccinated with WT-BCG showed increased IFN-γ secretion after stimulation

with dPT as compared to the non-immunized group (121). Surprisingly, WT-BCG vaccinated mice exhibited 80% of survival after a lethal dose challenge, suggesting that the non-specific protection induced by this vaccine can exhibit a protective effect on *B. pertussis* infections and consequently against whooping cough (121). Also, WT-BCG decreases some disease parameters in the mouse models for hRSV and hMPV infection (32). However, the non-specific protection induced by the wild-type vaccine was lower compared to the protection induced by the rBCG expressing hRSV and hMPV antigens, respectively. Mice immunized with the WT-BCG showed intermediate values of viral gene copies and infiltrating neutrophils in the lungs between those that were not immunized and those immunized with the specific rBCG vaccines (rBCG-N-hRSV, and rBCG-P-hMPV) (32). These results support the existence of trained immunity as a consequence of BCG vaccination. The development of a trained innate immune response after rBCG vaccination might be an advantageous scenario for preventing infections, due to the enhanced immune response elicited after immunization against homologous and heterologous pathogens.

DISCUSSION AND FUTURE CHALLENGES

The BCG vaccine has been used in humans for almost a 100 years, proving its immunogenicity and safety. However, it is still unclear whether recombinant BCGs can induce trained immunity after vaccination. Furthermore, the use of the BCG vaccine as a vector for the development of novel recombinant vaccines displays a series of advantages. It is stable, has a low cost of production and acts as an auto-adjuvant, promoting the generation of T_{H1} phenotypes in $CD4^+$ T cells that secrete high levels of IFN-γ and that are active against intracellular pathogens. Also, it generates $CD8^+$ T cell activation that mediates a cytotoxic response. In addition to protection against M. *tuberculosis*, a characteristic that draws much attention to this vaccine is its effect on decreasing infant mortality in a non-specific manner. This characteristic gives this vaccine a great advantage over other vaccines that confer protection only against the pathogen for which they were developed. Related to this, it was recently described that BCG induces a memory phenotype in innate immune cells, a phenomenon that is known as "trained immunity." Trained immunity confers non-specific protection against different pathogens, inducing upregulation of PPRs and the secretion of pro-inflammatory cytokines through epigenetic and metabolic reprogramming. This non-specific protection represents a great advantage when used in newborns since they still do not have a complete development of the adaptive immune system and have not been exposed to a wide range of harmful pathogens. Due to this, the training of innate immune cells conferred by BCG could be playing fundamental roles in helping the vaccinated individuals to respond to a wide variety of pathogens.

Trained immunity induced by BCG vaccination can be considered as a potential approach for improving vaccine development and effectiveness, due to the capacity to promote non-specific stimulation of PRRs in innate immune cells. Activation of these cells may contribute to the protection against different pathogens for which no specific vaccine yet exists. As for the case of the influenza A virus, the high mutation rate of this pathogen can impair the effectiveness of highly specific vaccines, thus the broad spectrum of pathogens to which trained immunity response could be a great advantage for the protection to such infectious agents (133, 134). Furthermore, viral infections such as hRSV have been shown to increase the host susceptibility to bacterial infections (135). Thus, vaccines capable of inducing trained immunity could potentially decrease the occurrence of coinfections. Besides, innate immune training strategies could also be applied to children, elderly or immunocompromised individuals that are unable to developing T or B cell-based specific immune responses (136–141). For these cases, the priming of the innate immune response could lead to a better immune response in cases of infections with a broad spectrum of pathogens, as trained immunity has been shown to respond well to viral, bacterial and fungal infections (96, 112, 114).

Even though BCG has been considered a good immunogenic vector, some factors need to be considered when using this bacterium to develop recombinant vaccines. One issue associated with recombinant BCG vaccines is the level of expression of the heterologous antigen (142). The selection of the vector is crucial, given that expression of the heterologous antigen can be mediated by integrative or replicative vectors. In the case of replicative vectors, as there can be more than one copy in a single mycobacterium, increasing the expression level of the antigen of interest (143). Despite this, integrative vectors are more stable and can promote a more stable expression of the antigen in time, which can, in turn, lead to a longer-lasting immune response (143). However, replicative vectors may have a risk of horizontal transfer to other bacteria present in the host, reducing the safety of the vaccine (144). As for the case of viral proteins, these pathogens use the host transcription and translation machinery to produce their proteins (145). Therefore, a potential drawback for using rBCG vaccines for viruses is the possibility that the expression of viral proteins by prokaryotic cells could proteins with variant conformations or altered epitopes (145). To address this problem, the most immunogenic peptides of the protein of interest could be identified and then cloned into a BCG to express only these peptides. In this way, the efficacy of the recombinant vaccine may be improved.

If recombinant BCGs can induce trained immunity after vaccination, we could think that trained immunity induced by rBCG could, in combination with the specific response, induce robust protection against the pathogen of interest. Determination of the development of trained immunity as a consequence of vaccination with recombinant BCG could represent another good advantage for this type of vaccine. Indeed, improving innate immunity represents an ideal complement for the cellular and/or humoral responses developed by rBCG.

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 Consequences of Mixed-Species Malaria Parasite Co-Infections in Mice and Mosquitoes for Disease Severity, Parasite Fitness, and Transmission Success

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The distributions of human malaria parasite species overlap in most malarious regions of the world, and co-infections involving two or more malaria parasite species are common. Little is known about the consequences of interactions between species during co-infection for disease severity and parasite transmission success. Anti-malarial interventions can have disproportionate effects on malaria parasite species and may locally differentially reduce the number of species in circulation. Thus, it is important to have a clearer understanding of how the interactions between species affect disease and transmission dynamics. Controlled competition experiments using human malaria parasites are impossible, and thus we assessed the consequences of mixed-species infections on parasite fitness, disease severity, and transmission success using the rodent malaria parasite species *Plasmodium chabaudi*, *Plasmodium yoelii*, and *Plasmodium vinckei*. We compared the fitness of individual species within single species and co-infections in mice. We also assessed the disease severity of single vs. mixed infections in mice by measuring mortality rates, anemia, and weight loss. Finally, we compared the transmission success of parasites in single or mixed species infections by quantifying oocyst development in *Anopheles stephensi* mosquitoes. We found that co-infections of *P. yoelii* with either *P. vinckei* or *P. chabaudi* led to a dramatic increase in infection virulence, with 100% mortality observed in mixed species infections, compared to no mortality for *P. yoelii* and *P. vinckei* single infections, and 40% mortality for *P. chabaudi* single infections. The increased mortality in the mixed infections was associated with an inability to clear parasitaemia, with the non-*P. yoelii* parasite species persisting at higher parasite densities than in single infections. *P. yoelii* growth was suppressed in all mixed infections compared to single infections. Transmissibility of *P. vinckei* and *P. chabaudi* to mosquitoes was also reduced in the presence of *P. yoelii* in co-infections compared to single infections. The increased virulence of co-infections containing *P. yoelii*

(reticulocyte restricted) and *P. chabaudi* or *P. vinckei* (predominantly normocyte restricted) may be due to parasite cell tropism and/or immune modulation of the host. We explain the reduction in transmission success of species in co-infections in terms of inter-species gamete incompatibility.

Keywords: malaria, mixed-species, virulence, co-infections, *Plasmodium yoelii*, *Plasmodium vinckei*, *Plasmodium chabaudi*

INTRODUCTION

Eight malaria parasite species are infectious to humans; namely, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale wallikeri*, *Plasmodium ovale curtisii*, *Plasmodium knowlesi*, *Plasmodium cynomolgi,* and *Plasmodium simium*. The latter three species are parasites of non-human primates, but also cause zoonotic malaria in humans (1–3). In large parts of the tropical world the ranges of at least some of these species overlap, they are often vectored by the same mosquitoes (4), and mixed-species infections are common (5–7).

Mixed species infections of human malaria parasites are well-documented in natural (8–12) and experimental [e.g., (13, 14)] settings. They are studied regarding diagnosis (15–17), treatment (18), immune response (19), virulence (12, 20), transmission (21–23), and in discussions of public health policy (6). The virulence of malaria infection is also of interest in the context of co-infection with other pathogens, such as HIV and *Schistosoma* (24–26).

The consequences of mixed-species infections on malaria disease and parasite fitness are incompletely understood. There is conflicting evidence from laboratory and field studies regarding the capacity of mixed-species infections to exacerbate (27) or ameliorate (14) disease. Furthermore, the mechanisms underlying the interactions between parasite species in mixed infections are complicated and multi-factorial, possibly involving both within-host competition (28), and cross-immunity (29).

Mixed species and mixed strain *Plasmodium* infections have been studied in primate (30) and rodent malaria parasite models (27, 28, 31, 32), as these enable the study of all parasite lifecycle stages including those that occur in mosquitoes. The rodent model is enhanced by the availability of multiple rodent malaria parasite species; namely, *Plasmodium yoelii*, *Plasmodium berghei*, *Plasmodium vinckei*, and *Plasmodium chabaudi*. For *P. yoelii* there are additionally several parasite strains that differ in virulence following inoculation of mice (33).

Previous studies on the consequences of mixed species infections for disease pathology using the rodent malaria parasites in mice have yielded conflicting and varied results. Snounou et al. found that the virulence of mixed-species infections, as measured by host mortality, was reduced compared to that of single infections of the constituent species (34) whilst Ramiro et al. found the opposite effect (27).

Here we describe the results of a series of experiments utilizing multiple strains of the rodent malaria parasite species *P. yoelii*, *P. chabaudi,* and *P. vinckei*, in which mixed infections of various combinations of species and strains were established and studied in both mice and mosquitoes. These rodent malaria parasite species display important phenotypic differences, specifically in their red blood cell type tropism, that impact on disease progression. The consequences of mixed species infections for disease severity in both hosts, parasite fitness, and transmission capacity were analyzed.

MATERIALS AND METHODS

Parasites, Mice and Mosquitoes

Four rodent malaria parasite strains, comprising three species, were used in these experiments; specifically, *P. chabaudi chabaudi* clone AJ, *P. chabaudi chabaudi* clone AS_{ED} (intermediate virulence, normocyte preference) (35), *P. yoelii* clone CU (non-virulent, reticulocyte restricted) (32), and *P. vinckei lentum* clone DS (non-virulent, normocyte preference) (36). These parasite lines were obtained from deep-frozen stocks kept at the University of Edinburgh (curated by Professors Richard Carter and David Walliker) and were originally isolated from thicket rats in Central Africa (35). Six-week-old female CBA mice, *Mus musculus*, were purchased from SLC Inc. (Shizuoka, Japan) and were used for all experiments. Mice were housed in a 12-h/12-h light/dark cycle at 24°C and fed with 0.05% para-aminobenzoic acid (PABA)-supplemented water to assist the growth of parasites. *Anopheles stephensi* mosquitoes were housed in a temperature- and humidity-controlled insectary at 23°C and 75% humidity. Mosquitoes used in the transmission experiments were maintained on 10% glucose solution supplemented with 0.05% PABA.

DNA Extraction and Real Time Quantitative PCR (qPCR)

To determine the proportion of each species in mixed infections, quantitative real time PCR (qPCR) was used to measure copy numbers of the merozoite surface protein 1 gene (*msp1*). DNA was extracted from infected mouse tail blood and infected mosquito midguts using an EZI DNA Investigator Kit (GIAGQN) according to the manufacturer's instructions. Quantitative PCR was performed on an ABI 7500 real-time PCR machine using a Power SYBR Green kit (Applied Biosystems, UK). Primers were designed based on a species-specific region of

msp1, as follows: *Py*CUmsp1F 5′CACCCTCAATAAACCCTGC-3′, *Py*CUmsp1R 5′-CGTGTACCAATACTTGAGTCAGAAC-3′; *Pv*DSmsp1F 5′-CAAGAAGCCTCACAACAAGAATCTA-3′, *Pv*DSmsp1R, 5′TGCTGGTTGGGCAGGTGCTGGA-3′, and *Pc*AJmsp1F 5′-GTACAAGAAGGAGCATCAGC-3′, *Pc*AJmsp1R 5′-GCGGGTTCTGTTGAGGCTCCT-3′. PCR assays were conducted on an AB7500 real-time PCR machine (Applied Biosystems, Japan) under the conditions: initial denaturation step of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and finally 61°C for 1 min. Copy numbers of *msp1* were quantified with reference to a standard curve generated from known numbers of plasmids containing the target sequence. As different parasite species have differing mean copy numbers of *msp1* per infected erythrocyte (due to different rates of DNA replication, differing numbers of merozoites per schizont, and differing propensities for multiple erythrocyte invasion), we normalized the proportion of each species in mixed infections by the copy numbers per infected erythrocyte calculated from single species infections. This methodology was also used to quantify the numbers of species-specific oocysts on the midguts of co-infected mosquitoes.

Experiments Involving the Monitoring of Virulence in Mice

Eight experimental groups of five mice each were set up to measure the effects of co-infections of parasite species on mice and to compare the growth of species in single and mixed species infections. Three of these groups were inoculated via intravenous (IV) injection with parasite infected red blood cells (iRBCs) of a single *P. c. chabaudi* clone AJ (hereafter referred to as *Pc*AJ), *P. c. chabaudi* AS_{ED} (*Pc*AS_{ED}), *P. y. yoelii* clone CU (*Py*CU) or *P. v. lentum* clone DS (*Pv*DS). The remaining four received mixtures of two species in equal numbers (*Pc*AJ+*Py*CU, *Py*CU+*Pc*AS_{ED}, *Py*CU+*Pv*DS and *Pc*AJ+*Pv*DS). Inocula were diluted in a solution of 50% fetal calf Serum (FCS) and 50% Ringer's solution (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl). Mice infected with single parasite species received 10^6 iRBCs, and co-infected mice received 10^6 of each component parasite species. It has been shown that a 2-fold difference in parasite numbers has a negligible effect on parasite dynamics and virulence (37).

Virulence was determined using the parameters of mortality, weight loss, and reduction in erythrocyte density, and was measured daily up to day 30 post-inoculation. Erythrocyte densities were counted using a Coulter Counter (Backman coulter, Florida) from 1:40,000 dilution of 2 μl whole blood sampled from tails in Isoton solution (Beckman coulter, Florida). Giemsa's solution stained thin blood smears from tail vein blood were monitored for parasitaemia for 30 days post-inoculation to assess the parasite replication rate. Whole blood samples (10 μl) were collected daily from day 1 to 30 into citrate saline, centrifuged briefly, and the erythrocyte pellet stored at −80°C prior to DNA extraction using an EZ1 DNA Investigator Kit (QIAGEN, Japan) and an EZ1 BioRobot (QIAGEN, Japan). Species specific qPCR based on the *msp1* gene was used to measure the proportions of each parasite in the mixed infections (32, 38). All experiments were performed twice.

Mosquito Transmission Experiments: Estimation of Mosquito Fitness and Parasite Species Transmission Capacity

Groups of mice were infected with single and mixed species infections of *Py*CU, *Pv*DS, and *Pc*AJ parasites (total six groups, each of five mice). On days 3 and 5 post-inoculation, individual groups of mosquitoes ($n = 40$ mosquitoes per group; 5–7 days post emergence from pupae) were fed on individual mice. Immediately following the feed, 20 mosquitoes from each group were pooled by mouse group into 12 cages, and egg bowls added 2 days later to allow the collection of eggs. These groups were monitored for longevity by counting dead mosquitoes daily up to day 60, and the numbers of larvae produced per mosquito were counted at day 5 post-hatching. Seven days later, 20 mosquitoes were removed from each group (57 groups total, as the number of mice fed from group *Pv*DS and *Pc*AJ + *Pv*DS was reduced to three and four, respectively for the day 5 feed) and the midgut oocyst burden recorded following dissection. Dissected midguts were stored at −80°C prior to DNA extraction for species proportion analysis by qPCR.

Statistical Analyses

All graphs were generated using GraphPad Prism 6 (GraphPad software Inc, USA). Comparison of survival curves was carried out using Log-rank (Mantel-Cox) tests. Multiple *t*-tests, corrected for multiple comparisons using the Holm-Sidak method, were used for comparing parasitaemia, erythrocyte density, weight loss, and parasite density of single and mixed infection in mice at all days during infection. Mann Whitney tests were carried out for cumulative parasite density, mosquito infection, and analysis of oocysts per gravid mosquito. *P*-values of below 0.05 were considered significant.

RESULTS

Mixed Species Parasite Infections Involving a Reticulocyte Specialist and a Normocyte Specialist Are More Virulent and Cause Greater Host Mortality Than Single Species Infections in Mice

Infection parameters for single and mixed infections involving *Py*CU (reticulocyte restricted) and either *Pv*DS, *Pc*AJ, or *Pc*AS_{ED} (normocyte preference) are summarized in **Table 1**. *Py*CU and *Pv*DS are not lethal in single species infections and only the intermediately virulent species *P. chabaudi* (*Pc*AJ and *Pc*AS_{ED}) caused death of mice in single infections, with 40% mortality occurring between days 9 and 13 post-infection (PI) for both *Pc* strains. In contrast, mixed-species co-infections of *Py*CU with either *Pv*DS, *Pc*AJ, or *Pc*AS_{ED} resulted in highly virulent infections with 100% mortality (**Figures 1A–C**).

Mixed species infections resulted in higher parasitaemia than either of their constituent species in single infections (**Figure 1D**) and peak parasitaemia occurred on the same day PI as the more virulent of the constituent species; except for *Py*CU + *Pv*DS in which peak parasitaemia occurred between days 8 and 11, compared to the *Pv*DS single

TABLE 1 | Infection parameters for single and mixed species infections of *Plasmodium yoelii* CU with *Plasmodium vinckei* DS, *Plasmodium chabaudi* AJ, and *Plasmodium chabaudi* AS_{ED}.

Strain	*Py*CU	*Pv*DS	*Pc*AJ	*Pc*AS_{ED}	*Py*CU + *Pv*DS	*Py*CU + *Pc*AJ	*Py*CU + *Pc*AS_{ED}
RBC invasion preference	**Reticulocytes**	**Normocytes**	**Normocytes and reticulocytes**	**Normocytes and reticulocytes**	/	/	/
Peak Parasitaemia % (day pi)	52.57 ± 1.89 (18–19, 25)	36.44 ± 0.54 (6–7)	63.85 ± 4.94 (7)	59.91 ± 1.03 (6, 7)	75.78 ± 1.72 (8, 10–11)	59.96 ± 3.56 (7–8)	64.32 ± 3.43 (7, 9)
Cumulative parasitaemia	544.74 ± 80.00	127.94 ± 6.32	245.55 ± 14.83	190.21 ± 7.49	281.52 ± 26.66	218.39 ± 16.87	188.53 ± 9.45
Mortality (day pi)	0%	0%	40% (10, 13)	40% (9, 10)	100% (9, 11, 12)	100% (9, 10)	100% (9–11)
Max. weight loss, g (day pi)	1.26 ± 0.44 (19–21, 27)	2.49 ± 1.00 (8, 10)	4.06 ± 0.54 (9–13)	3.54 ± 0.38 (8, 9)	4.26 ± 0.66 (8, 9–12)	2.93 ± 0.30 (8–9)	2.92 ± 0.18 (8, 10)
Min. RBC count, RBC/ml (day pi)	$1.61 \times 10^9 \pm 0.13 \times 10^9$ (21, 26, 28)	$3.61 \times 10^9 \pm 0.05 \times 10^9$ (8, 9)	$1.51 \times 10^9 \pm 0.21 \times 10^9$ (9)	$1.41 \times 10^9 \pm 0.13 \times 10^9$ (7, 8)	$1.60 \times 10^9 \pm 0.04 \times 10^9$ (8–11)	$1.65 \times 10^9 \pm 0.22 \times 10^9$ (8, 9)	$1.24 \times 10^9 \pm 0.08 \times 10^9$ (7, 8)

infection, in which peak parasitaemia occurred at days 6–7 (**Figures 1D–F**). Host mortality in mixed species infections occurred at peak parasitaemia and was presumably caused by anemia resulting from an inability to clear parasites from the blood.

During the latter stages of the infection mixed species infections involving *Pv*DS and *Py*CU resulted in lower erythrocyte densities (**Figures 1G–I**) and greater weight loss (**Figures 1J–L**) compared to either of the constituent species in single infections. This increased pathology was linked to the inability of mice to control parasitaemia.

Mixed Species Parasite Infections Involving Two Normocyte Specialists Result in Protracted Parasitaemia, but Not Increased Virulence

Plasmodium vinckei DS and *P. chabaudi* AJ are both normocyte invading parasites. *Pc*AJ is of moderate virulence, causing rapid and severe anemia and weight loss during the first 10 days of infection when parasitaemia rises, and results in 40% host mortality (**Table 2** and **Figure 2A**). *Pv*DS is a much less virulent parasite, causing less severe weight loss and milder anemia, and is never lethal (**Table 2** and **Figure 2A**). The combination of these two parasites in a mixed infection results in 50% host mortality, and a pathology consistent with that of *Pc*AJ, the more virulent of the two species (**Table 2** and **Figure 2A**). However, the *Pc*AJ+*Pv*DS infection results in three distinct parasitaemia peaks, compared to the two peaks produced by the single species, and parasitaemias persisted up to the last day of the experiment (day 30), compared to clearance by day 26 in the single species infection (**Figure 2B**). The *Pc*AJ+*Pv*DS infection also displayed a sharper decline in parasitaemia following the first peak (**Figure 2B**), and this was associated with a quicker recovery from anemia and weight loss between days 10 and 15 (**Figures 2C,D**).

Co-Infection of *P. yoelii* With Either *P. chabaudi* or *P. vinckei* Results in Reduced Parasite Density of *P. yoelii*, and Protracted Peak Parasitaemia of *P. chabaudi* and *P. vinckei*

To understand how mixed parasite species infection influences the fitness of the species involved, we measured the parasite density (numbers of parasites per ml of mouse blood) through time of individual species in single and mixed infections. The relative proportions of each species within mixed infections were measured at 24-h intervals by species specific qPCR.

In mixed infections composed of *Py*CU and *Pv*DS, *Pv*DS dominated the infection from days 4 to 10 PI (**Figure 3A**), at which point *Py*CU became dominant. There was an increase in the proportion of *Py*CU in the infection from day 8 (7%) until host mortality at day 12 PI (50%). Analysis of species-specific parasite density in this co-infection revealed that *Py*CU was suppressed throughout the infection, while the growth of *Pv*DS was enhanced (**Figures 3D,E**).

This enhancement occurred during the latter stages of the infection (days 8–12), the time point at which *Pv*DS is cleared during single infections. This suggests that the presence of *Py*CU, whose growth in a co-infection does not differ significantly from that observed in a single species infection, facilitates the persistence of *Pv*DS for an extended period after which it would normally be cleared. This inability to clear *Pv*DS, combined with the standard increase in *Py*CU parasitaemia, leads to hyper-parasitaemia with severe anemia in co-infected mice, and results in host death.

Enhancement of the parasite density of the normocyte-restricted parasite species was also observed in the latter stages of mixed species infections composed of *Py*CU and *Pc*AJ. In this case, *Pc*AJ dominates *Py*CU throughout the infection, with complete exclusion of the latter species observed at the end of the co-infection (**Figure 3B**). Mice died at days 9 and 10 pi, at which point the parasite density of *Py*CU was significantly suppressed

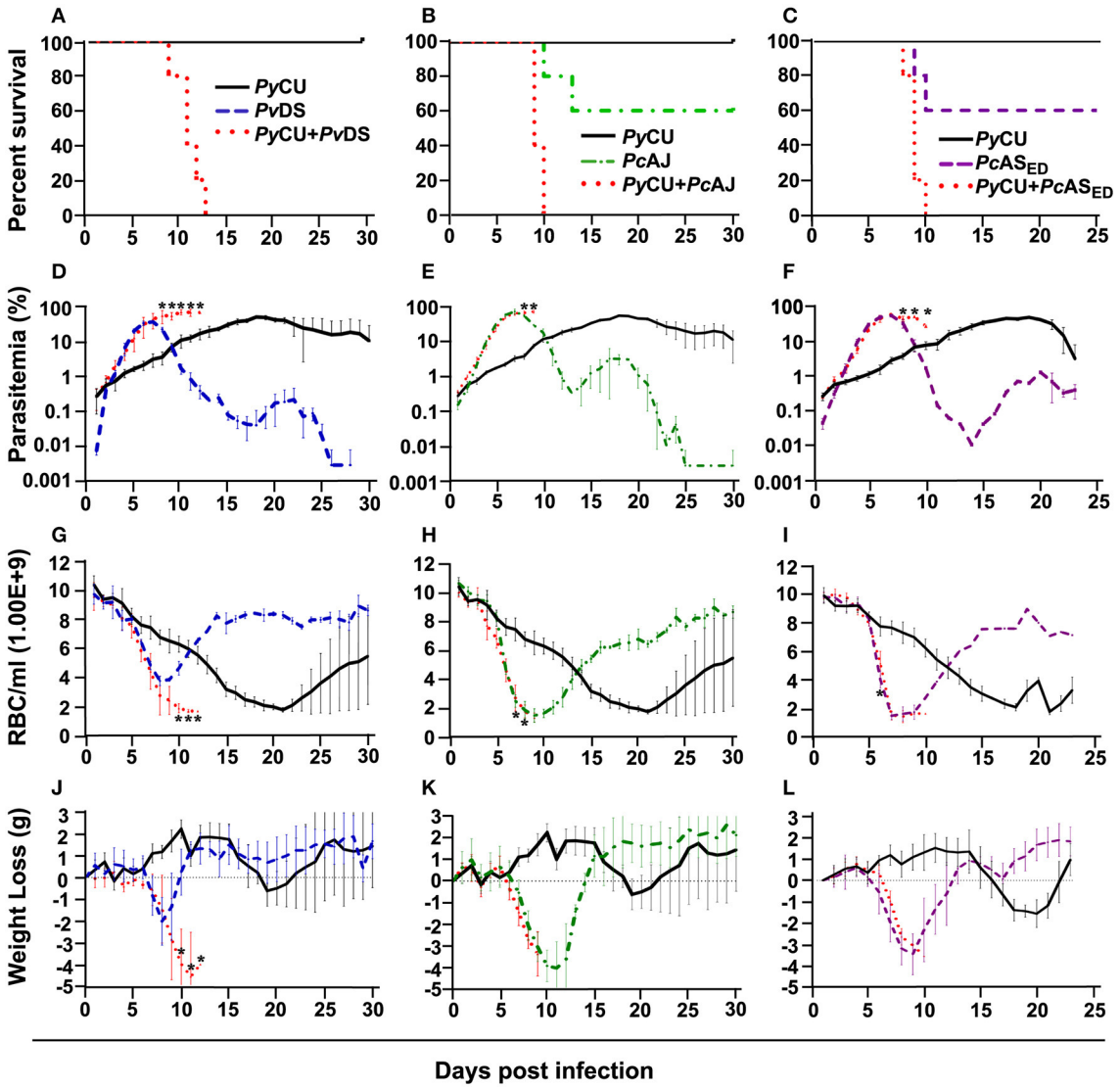

FIGURE 1 | Percent survival **(A–C)**, parasitaemia **(D–F)**, erythrocyte density **(G–I)**, and weight loss **(J–L)** of mice infected with *Plasmodium yoelii* CU (black), *Plasmodium vinckei* DS (blue), *Plasmodium chabaudi* AJ (green), and *Plasmodium chabaudi* AS_{ED} (purple) species in single and mixed infections (red). Mice were inoculated via intravenous injection of 1×10^6 infected red blood cells of either single or mixed species of the above parasites and followed for 25–30 days to determine mortality, live-body weight, erythrocyte density, and parasitaemia. Data points indicate the mean value for five mice in each experimental group and error bars indicate the standard error of the mean (SEM). An asterisk represents statistically significant differences between mixed infections compared with both single infections (Multiple *t*-tests, with the assumption that all rows are sampled from populations with the same scatter and corrected for multiple comparisons using the Holm-Sidak method). Parasitaemias of *Py*CU and *Pv*DS in mixed-infections were significantly different between days 8 and 12 post infection (PI) compared with the same strains in single infections **(D)**; *Py*CU and *Pc*AJ mixed-infections were significantly different to single infections of the same strains on days 8 and 9 PI **(E)**; and *Py*CU and *Pc*AS_{ED} mixed-infections were significantly different from single infections between days 8 and 10 PI **(F)**. Erythrocyte densities of *Py*CU and *Pv*DS mixed-infections were significantly different from single infections on day 10 to 12 PI **(G)**; *Py*CU and *Pc*AJ mixed-infections were significantly different on days 7 and 8 PI **(H)**; while mixed infections of *Py*CU and *Pc*AS_{ED} were significantly different from single infections only on day 6 PI **(I)**. Mice infected with *Py*CU+*Pv*DS mixed-infections lost significantly more weight compared to single infections from day 10 to 12 **(J)**. Mice in the groups infected with mixed infections of *Py*CU+*Pc*AJ suffered from significantly reduced erythrocyte density compared to mice in single infection groups (Two-way RM ANOVA measured mixed effects model, $P = 0.043$, $F = 5.7$, DFn = 1, DFd = 8). Detailed statistical values relating to significance are given in **Supporting Table 1**. Experiments were repeated twice, data is from one representative experiment.

compared to single infections (**Figure 3D**). In contrast, there was little difference in the parasite density of *Pc*AJ in a mixed infection with *Py*CU compared to a single infection during the first 8 days of the co-infection. However, as seen in the *Pv*DS+*Py*CU infection (**Figure 3E**), the usual reduction in parasitaemia observed at day 8 in *Pc*AJ single infections was not observed in co-infections with *Py*CU (**Figure 3F**), suggesting again that presence of *Py*CU in a co-infection impairs the ability

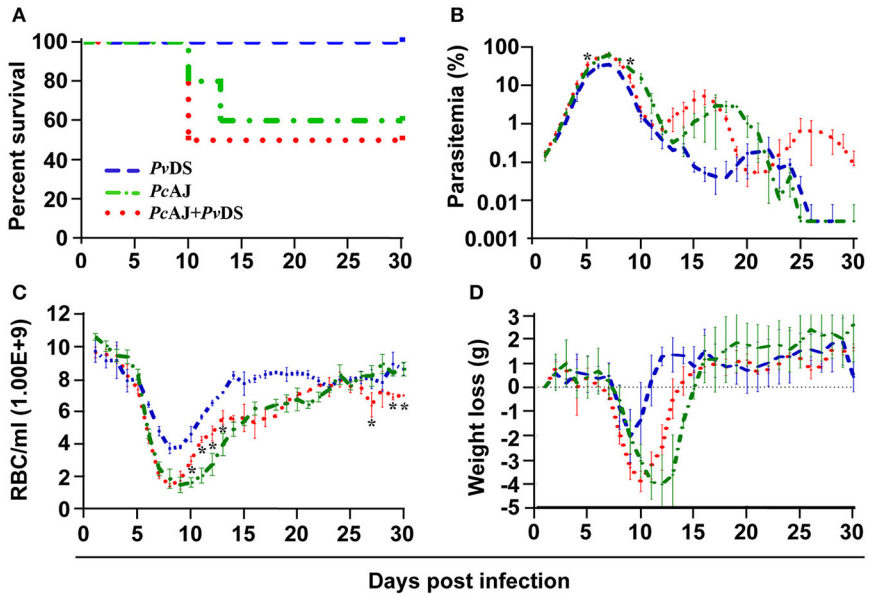

FIGURE 2 | Percentage survival **(A)**, parasitaemia **(B)**, erythrocyte density **(C)**, and weight loss **(D)** of infected mice with *Plasmodium vinckei* DS (blue), *Plasmodium chabaudi* AJ (green) single or mixed infections (red). Mice were inoculated via intravenous injection of 1 x 10^6 infected red blood cells of either single or mixed species of the above parasites and followed for 30 days to determine mortality, live-body weight, erythrocyte density, and parasitaemia. Data points indicate the mean value for mice of each experimental group and error bars indicate the SEM. An asterisk represents statistically significant difference of mixed infections compared with both single infections (Multiple *t*-tests, with the assumption that all rows are sampled from populations with the same scatter and corrected for multiple comparisons using the Holm-Sidak method). Mice infected with mixed-infections developed statistically significantly higher parasitaemia on days 5 and 9 PI compared with both single infections **(B)**. Mice infected with mixed-infections had significantly lower erythrocyte densities on days 10 to 13, 27, 29, and 30 PI compared with both single infections **(C)**. Considering the entire time course of the infections, the parasitaemia of mice infected with single infections of *Pv*DS were significantly lower than those of mice infected with *Pv*DS + *Pc*AJ mixed-infections (two-way RM ANOVA measured mixed effects model, $F = 218.8$, DFn = 1, DFd = 3, $P = 0.0007$). The erythrocyte density of mice infected with *Pv*DS + *Pc*AJ mixed-infections was significantly lower than those infected with single infections of *Pv*DS (two-way RM ANOVA measured mixed effects model, $F = 123$, DFn = 1, DFd = 3, $P = 0.0016$). Detailed statistical values relating to significance are given in **Supporting Table 1**. Experiments were repeated twice, data is from one representative experiment.

of the host to control the growth of the normocyte-invading parasite species.

Cumulative parasite densities were calculated for each parasite species in single and mixed infections as proxy measures of parasite productivity throughout the infection. There was a dramatic reduction in cumulative parasite density for *Py*CU in the mixed infections with both *Pc*AJ and *Pv*DS (**Figure 3G**). In the mixed infections involving *Pc*AJ and *Pv*DS, both of which preferentially invade normocytes, there was a dramatic reduction in cumulative parasite density for *Pv*DS throughout the co-infection compared to single infection (**Figure 3H**). There was also a slight reduction in cumulative parasite density of *Pc*AJ (**Figure 3I**), a reflection of lower productivity during the latter stages of the infection. *Pc*AJ is the more virulent of the two species and dominates the co-infection between days 5 and 15, when no *Pv*DS could be detected by qPCR. However, *Pv*DS resurges at day 15, and competitively excludes *Pc*AJ by day 25.

TABLE 2 | Infection parameters for single and mixed species infections of *Plasmodium vinckei* DS and *Plasmodium chabaudi* AJ.

Strain	*Pv*DS	*Pc*AJ	*Pc*AJ + *Pv*DS
RBC invasion preference	**Normocytes**	**Normocytes and reticulocytes**	**/**
Peak parasitaemia % (day pi)	36.44 ± 0.54 (6–7)	63.85 ± 4.94 (7)	65.67 ± 2.28 (6–7)
Cumulative parasitaemia	127.94 ± 6.32	245.55 ± 14.83	240.15 ± 6.07
Mortality (day pi)	0%	40% (10, 13)	50% (10)
Max. weight loss, g (day pi)	2.49 ± 1.00 (8, 10)	4.06 ± 0.54 (9–13)	3.98 ± 0.12 (9–10)
Min. RBC count, RBC/ml (day pi)	3.61 × 10^9 ± 0.05 × 10^9 (8, 9)	1.51 × 10^9 ± 0.21 × 10^9 (9)	1.46 × 10^9 ± 0.05 × 10^9 (8, 9)

Pre-exposure of Mice to *P. yoelii* Does Not Enhance the Virulence of *P. vinckei* Infection

As the presence of *P. yoelii* in mixed infections with either *P. chabaudi* or *P. vinckei* results in an inability to clear the latter two species, resulting in host death, we wondered whether an immune response specific to *P. yoelii* could adversely affect the establishment of an effective immune response against *P. chabaudi* or *P. vinckei*. To test this, we pre-immunized mice with *Py*CU parasites by exposure to the parasite for 8 days followed by clearance with the anti-schizontal drug mefloquine (MF). Two

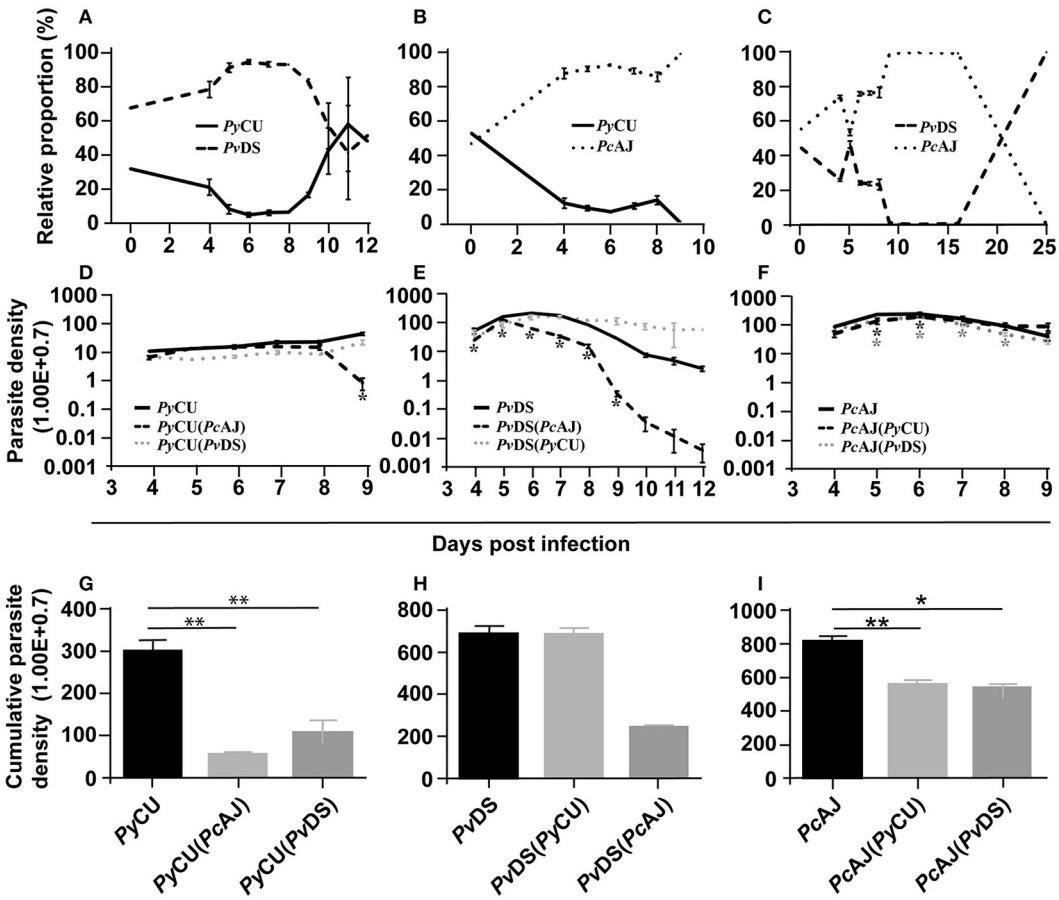

FIGURE 3 | The relative proportions of *Plasmodium yoelii* CU, *Plasmodium vinckei* DS, and *Plasmodium chabaudi* AJ in mixed infections **(A–C)**, the parasite density of each species in single and mixed infections **(D–F)**, and cumulative parasite density of each species in either single or mixed infections **(G–I)**. The relative proportion of each species in combination with each other **(A–C)** was measured by qPCR quantification using primers specific to a region of the *msp1* gene of each species. Copy numbers of parasite *msp1* were quantified with reference to a standard curve generated from known numbers of plasmids containing the same gene sequences. The average copy numbers per iRBC were generated by copy numbers and parasite densities of each single species infections on day 6 PI. Data points indicate the mean value for 3–5 mice in each experimental group and error bars indicate the SEM. The parasite densities (number of blood stage parasites per mL blood) are shown in **(D–F)**. **(D)** Shows the parasite densities of *Py*CU single infection or in mixed infection with *Pv*DS or *Pc*AJ. Parasite densities of PvDS and PcAJ are given in **(E,F)**, respectively. An asterisk represents significant differences ($P < 0.01$) in parasite density in single infections compared with mixed-infections. Cumulative parasite densities are shown in **(G–I)**. The cumulative parasite density of *Py*CU in single infections were significantly higher than *Py*CU in mixed-infections with *Py*AJ and *Pv*DS **(G)**. Similarly, the cumulative parasite density of *Pc*AJ in single infections was significantly higher than in mixed infections with *Py*CU or *Pv*DS **(I)**. Detailed statistical values relating to significance are given in **Supporting Table 1**. Experiments were repeated twice, data is from one representative experiment.

weeks later, when all MF had cleared from the host, mice were challenged with *Pv*DS. In contrast to the patterns observed in *Py*Cu+*Pv*DS co-infections, there was no evidence of increased virulence of *Pv*DS infections in mice pre-exposed to *Py*CU (**Figure 4A**), with no significant enhancement of parasite density (**Figure 4B**), anemia (**Figure 4C**), or weight loss (**Figure 4D**) occurring at any stage during the infection.

The Increased Virulence of Mixed Species Infections of *P. yoelii* and *P. vinckei* Is Abrogated When *P. vinckei* Is Added to an Established *P. yoelii* Infection

When both *Py*Cu and *Pv*DS are inoculated into mice contemporaneously, the resulting co-infection is consistently lethal, in contrast to the zero-mortality associated with the constituent single species infections. This lethality results from the inability of mice to clear the *Pv*DS parasites from the circulation following peak parasitaemia. To determine if increased virulence is dependent on the timing of the introduction of the co-infecting species, we first inoculated mice with *Py*CU and introduced *Pv*DS 7 days later. The co-infection caused 25% mortality, compared to no mortality in single species infections (**Figure 5A**). In this case, the co-infection parasitaemia did not differ significantly from that of a single infection of *Py*CU for most of the infection duration, except for the last 2 days of sampling (days 22 and 23), when the parasitaemia was higher in the co-infection (**Figure 5B**). This increased parasitaemia toward the latter stages of the infection did not result in lower erythrocyte density (**Figure 5C**); however,

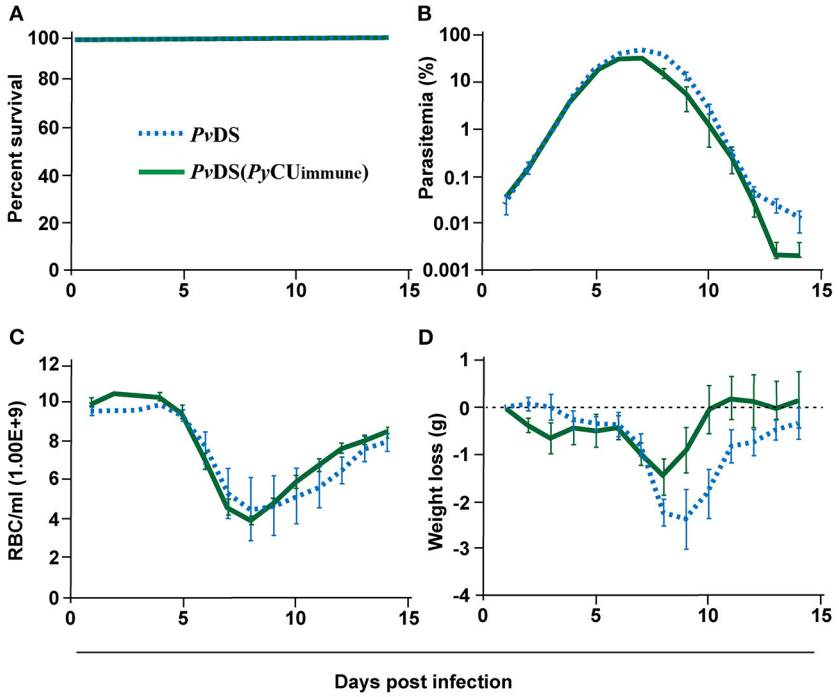

FIGURE 4 | Percentage survival **(A)**, parasitaemia **(B)**, erythrocyte density **(C)**, and weight loss **(D)** of mice infected with *Plasmodium vinckei* DS (*Pv*DS). Mice in the *Pv*DS (*Py*CU immune) group were inoculated intravenously (IV) with 1 × 10^6 infected red blood cells (iRBCs) of *Py*CU, treated 8 days later with mefloquine for 5 days, and then 15 days later intravenously challenged with 1 × 10^6 *Pv*DS iRBCs. Data points indicate the mean value for 5 mice in each experimental group and error bars indicate the SEM. Detailed statistical values relating to significance are given in **Supporting Table 1**.

it did cause significantly greater weight loss in co-infected animals during this period (**Figure 5D**).

Measuring the relative proportions and parasite densities of the constitute species in the co-infection and comparing them to single infections revealed that *Py*CU dominates the infection over *Pv*DS, excepting days 7 and 8 (**Figure 5E**). In contrast to the situation observed with the simultaneous inoculation of the two species, there was no significant reduction in the parasite density of *Py*CU, but there was a reduction in the parasite density of *Pv*DS when *Py*Cu was inoculated 1 week prior to *Pv*DS (**Figures 5F–I**).

The Consequences of Mixed Species Infections in the Mosquito Vector

We additionally sought to describe the impact of mixed species infections on transmission to mosquitoes. Specifically, we fed *Anopheles stephensi* mosquitoes on mice with single or mixed species infections and measured: (i) the proportion subsequently infected, and (ii) the severity of this infection (number of oocysts); (iii) the longevity of infected mosquitoes; and (iv) the number of larvae they produced following a blood meal. Finally, we compared the transmission success, defined as the average number of oocysts produced per blood fed mosquito, of each parasite species in mixed or single infections.

Mixed Species Infections Do Not Result in Significantly Different Infection Parameters in Mosquitoes

To determine whether mixed species infections result in altered mosquito infectivity rates and infection loads compared to single species infections, mosquitoes were fed on anesthetized mice infected with single, or mixed infections of *Pc*CU, *Pv*DS, and *Pc*AJ. As these species differ in the timing of their gametocyte production, with *Py*CU at its most infectious to mosquitoes on day 3 PI and *Pc*AJ and *Pv*DS more infectious on day 5 pi, we conducted mosquito feeds on both these days. *Py*Cu was the most infective single species on day 3 and 5, followed by *Pv*DS and finally *Pc*AJ (**Figures 6A–C**). Mixed species infections did not result in higher proportions of mosquitoes being infected than the most infective constituent species in a single infection (**Figures 6A–C**). Similarly, in co-infections containing the highly infectious *Py*CU species, the oocyst burdens of mixed species infections were not significantly different from that of *Py*CU in mosquitoes fed on mixed infections on either day 3 or 5 PI (**Figures 6D,E**). Mixed species infections of *Pc*AJ and *Pv*DS resulted in significantly lower oocyst burdens than the most infectious constituent single species (*Pv*DS), but only in mosquitoes fed on day 5 of the infection (**Figure 6F**).

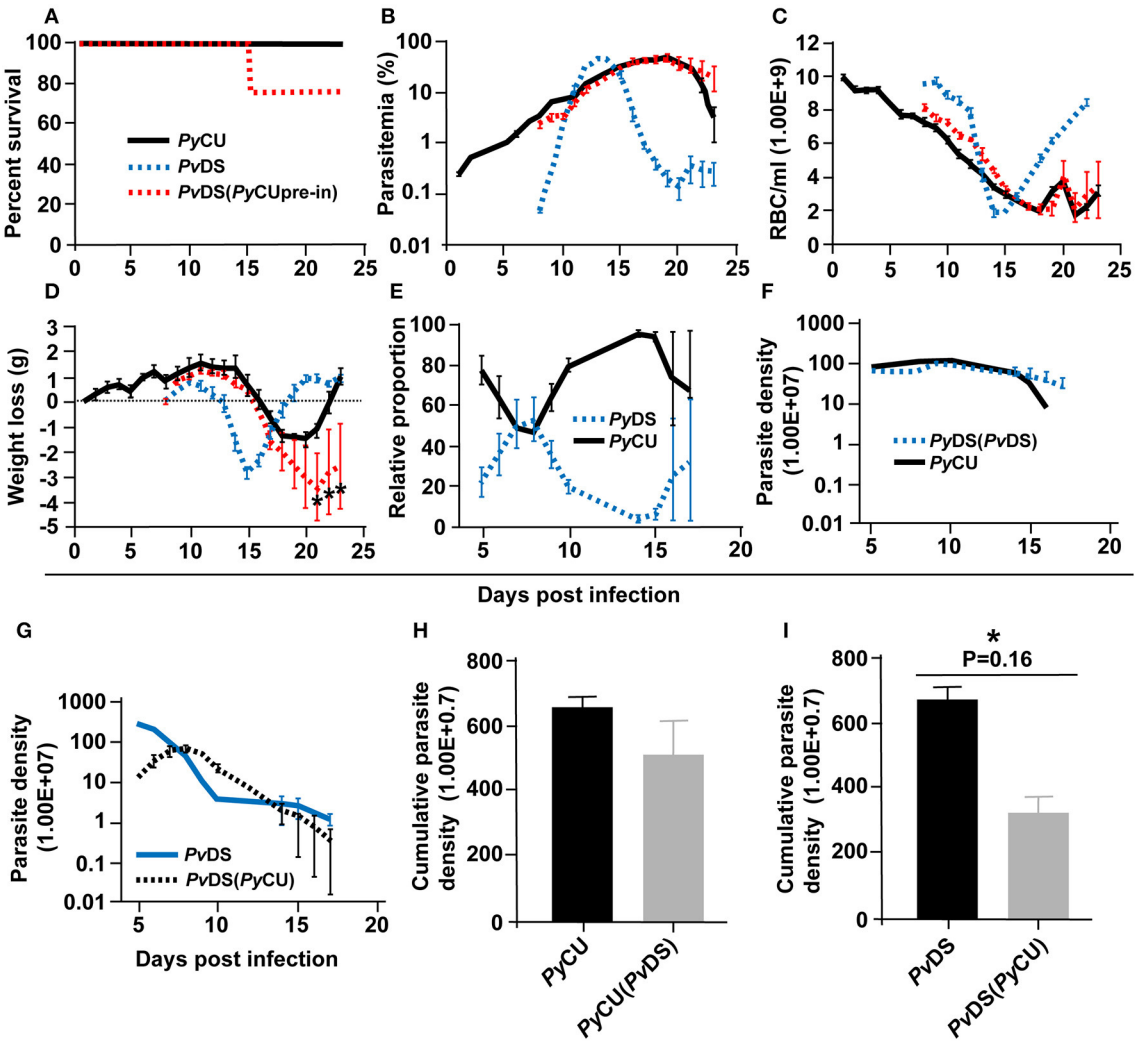

FIGURE 5 | Percentage survival **(A)**, parasitaemia **(B)**, erythrocyte density **(C)**, and weight loss **(D)** of mice infected with *Plasmodium yoelii* CU (*Py*CU) and *Plasmodium vinckei* DS (*Pv*DS) in single and mixed infections. Mice in the mixed-infection group were inoculated intravenously (IV) with 1×10^6 *Py*CU infected red blood cells (iRBCs) 7 days prior to IV inoculation with 1×10^6 *Pv*DS iRBCs. Data points indicate the mean value for 4–5 mice in each experimental group and error bars indicate the SEM. Asterisks indicate statistically significant differences between mice with mixed-species infections compared with both single infections (Multiple *t*-tests, with the assumption that all rows are sampled from populations with the same scatter and corrected for multiple comparisons using the Holm-Sidak method). Mice infected with mixed-infections lost significantly more weight during the latter stages of the infection than mice infected with single species infections (days 21–23 PI, **D**). The relative proportion of each species in mixed infections **(E)** was measured by qPCR quantification of the *msp1* gene. The average copy number per iRBC was generated with reference to copy numbers and parasite densities of each single species infections on day 6 PI. The parasite densities of *Py*CU and *Pv*DS in single or mixed infections are shown in **(F,G)**. A Mann Whitney test shows that the cumulative parasite density of *Pv*DS in single infections is significantly higher than that of *Pv*DS in a mixed infection with *Py*CU **(I)**, whereas that of *Py*CU is unaffected when in a mixed infection with *Pv*DS **(H)**. Detailed statistical values relating to significance are given in **Supporting Table 1**.

Co-Infections of Malaria Parasite Species Do Not Adversely Affect Mosquito Longevity or Capacity to Produce Larvae

To ascertain whether mixed species infections of mosquitoes were more virulent than single species infections, we measured longevity and larvae production in mosquitoes fed on single or mixed infections. In accordance with the observation that mixed species infections did not result in higher burdens of infection, mosquitoes infected with two parasite species in a co-infection did not display reduced longevity (**Figures 7A–C**), median survival time (**Figure 7D**), or reduced fitness (measured as the number of larvae produced per blood-fed mosquito) (**Figure 7E**).

Mixed Species Infections Can Affect the Transmission Capacity of the Constituent Species

To determine whether mixed infections can affect the transmission capacity of constituent species, the relative proportion of each species in mixed infections in mosquitoes was

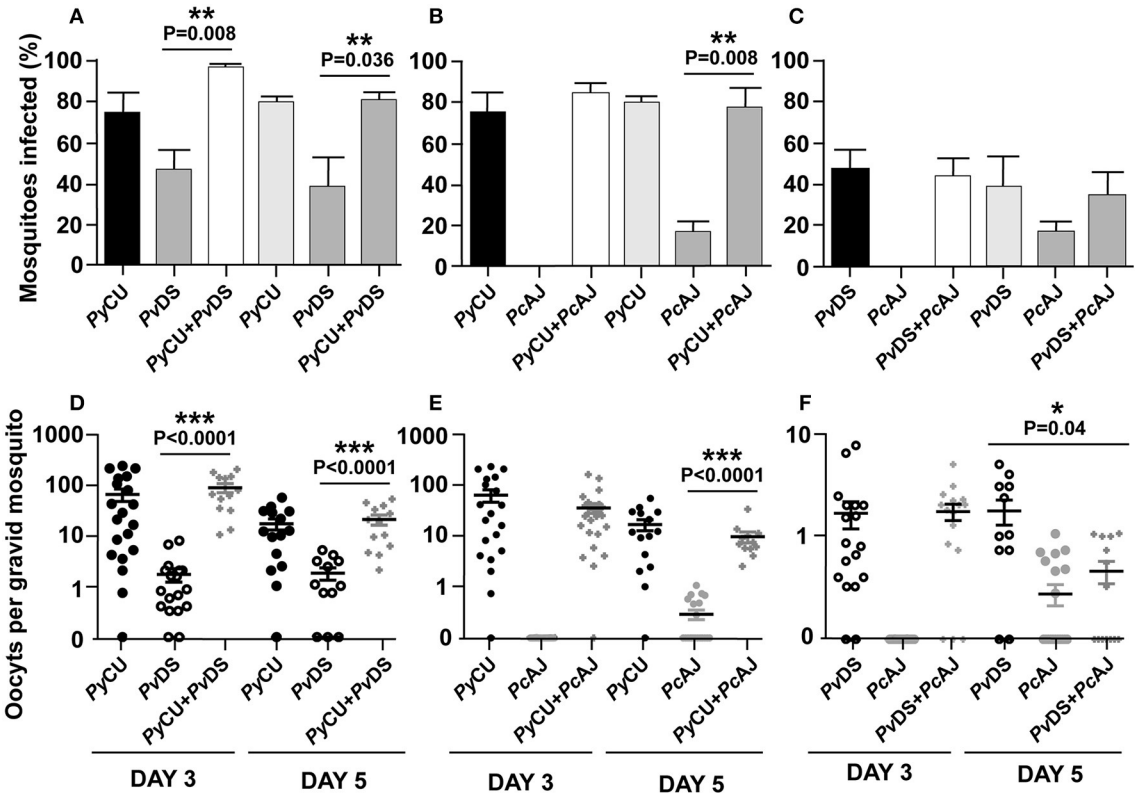

FIGURE 6 | Transmission capacity of Plasmodium yoelii CU (PyCU), Plasmodium vinckei DS (PvDS), and Plasmodium chabaudi AJ (PcAJ) in single or mixed infections. The mean percentage of mosquitoes infected with oocysts were calculated following feeding on PyCU, PvDS, and PcAJ either in single or mixed infections in mice on days 3 and 5 PI **(A–C)**. The numbers of oocysts per gravid mosquito are given for all groups **(D–F)**. Only gravid mosquitoes were considered blood-fed and included in the analysis. Statistical analysis was performed using Mann Whitney tests. Detailed statistical values relating to significance are given in **Supporting Table 1**.

measured using qPCR on DNA extracted from mosquitoes with known oocyst numbers and compared to the numbers produced in single infections.

The numbers of oocysts produced by the highly infectious *Py*CU did not differ significantly between mosquitoes fed on single and mixed species infections (**Figure 8A**). *Pv*DS produced fewer oocysts in mixed infections with *Pc*AJ and *Py*CU than in single infections, although the effect was only statistically significant on day 5 in a mixed infection with *Py*CU (**Figure 8B**). *Pc*AJ also suffered a reduced transmission capacity in mixed infections, with significant reductions in oocyst numbers measured in mosquitoes fed on mixed infections with either *Pv*DS or *Py*CU on day 5, compared to those fed on single infections (**Figure 8C**). The transmission of *Pc*AJ to mosquitoes was completely blocked in mixed infections containing *Py*CU (**Figure 8C**).

DISCUSSION

Our results indicate that the interactions between malaria parasites co-infecting the same host can have dramatic consequences for the severity of the disease they cause. We found that when two parasite species, *P. yoelii* and *P. vinckei*, which on their own cause mild and transient disease concurrently infect the same host, the disease outcome is radically altered resulting in 100% host mortality within 15 days. This same outcome of 100% host mortality was observed in co-infections consisting of *P. yoelii* and the more virulent (but rarely lethal) *P. chabaudi*.

There are precedents for this result; Bafort (39) suggested that mixed species might increase the virulence of infections (39). Richie (9) reported that patent *P. chabaudi* infections increased their parasitaemia and duration when mixed with *P. yoelii* (9), and McGhee (40) also described a higher peak for one of the species in mixed infections (40). In contrast, Snounou et al. found a *protective* effect of mixing *P. yoelii* with *P. vinckei* or *P. chabaudi*, with less mortality in the mixed species infection groups compared to the single infection groups (34). Simlarly, Voza et al. reported that the addition of co-infecting *P. yoelii* to a *P. berghei* infection prevented the establishment of cerebral malaria, whereas this protection was not observed when co-infections with *P. vinckei* were induced (41).

More recently, Ramiro et al. (27) described increased virulence in mixed infections of *P. chabaudi* and *P. yoelii*, which they attributed to an increase in reticulocytaemia leading to enhancement of *P. yoelii* (which is reticulocyte restricted) in the mixed infections. Our results, however, are in agreement with those of Richie (9), and suggest that it is the normocyte-restricted

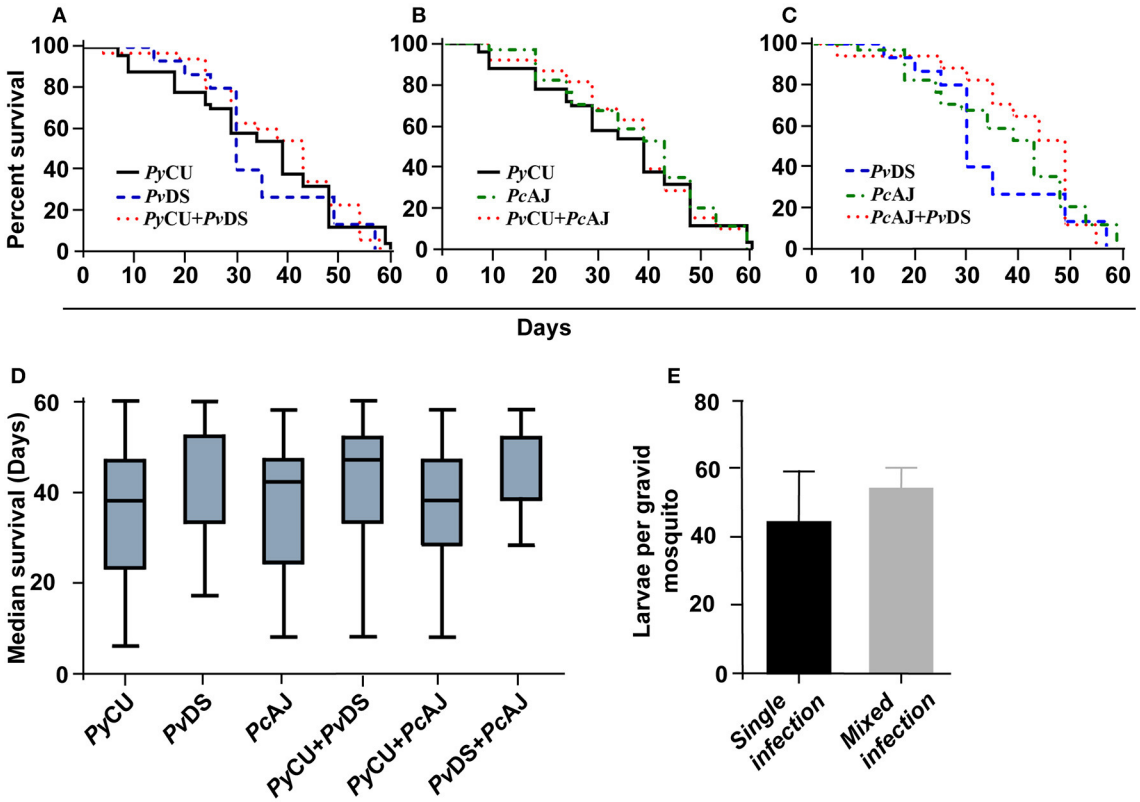

FIGURE 7 | Survival **(A–D)** and larvae per mosquito **(E)** of mosquitoes infected with single and mixed species infections. Mosquito survival curves, infected with either single species or mixed infection of *Plasmodium yoelii* CU, *Plasmodium vinckei* DS, and *Plasmodium chabaudi* AJ are shown in **(A–C)**. Longevity of infected mosquitoes was observed until day 60 after the blood meal and the number of dead mosquitoes were recorded every 5 days. Boxplots indicate median survival and first and third quartiles, and whiskers are the same quartiles ± (1.5 × interquartile range) **(D)**. The numbers of larvae from infected mosquitoes with single or mixed infections were recorded and no significant difference was observed. Detailed statistical values relating to significance are given in **Supporting Table 1**.

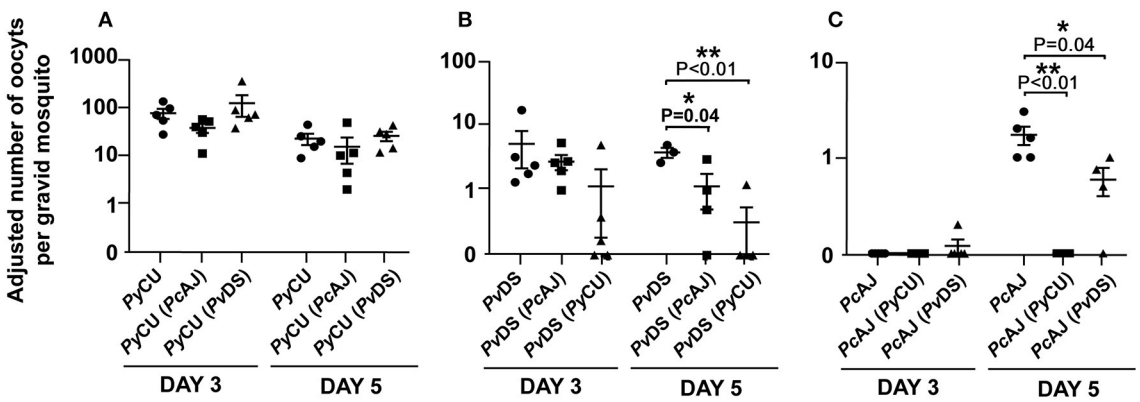

FIGURE 8 | Adjusted mean number of oocysts of *Plasmodium yoelii* CU (*Py*CU), *Plasmodium vinckei* DS (*Pv*DS), and *Plasmodium chabaudi* AJ (*Pc*AJ) in single or mixed species infections per gravid mosquito. Data points represent the mean oocyst burden of mosquitoes fed on individual mice ($n = 5$ per group). There was no statistical difference between *Py*CU in single and in mixed infections with either *Pc*AJ or *Pv*DS **(A)**, *Pv*DS was suppressed when mixed with *Py*CU on day 5 PI **(B)** and *Pc*AJ was suppressed in mixed infections with *Py*CU and *Pv*DS on day 5 PI **(C)**. Detailed statistical values relating to significance are given in **Supporting Table 1**.

parasite that is enhanced in mixed infections with *P. yoelii*, a result we observed both in the case of two strains of *P. chabaudi* and one of *P. vinckei*. We contend, therefore, that increasing reticulocytaemia does not explain the increase in virulence of mixed strain infections. This is supported by the fact that we also observed increased virulence (in terms of persistence of

infection and decreased red blood cell density) in mixed-species infections composed of *P. chabaudi* and *P. vinckei*, both of which predominantly infect normocytes.

We found that the time at which the constituent species of a mixed-species infection were introduced to the host had a significant impact on disease outcome. Most studies on mixed malaria parasite species infections in mice introduce the constituent species contemporaneously (27, 28). For example, the parasite species combination *P. yoelii* and *P. vinckei* causes 100% mortality when introduced to mice contemporaneously. We found that when *P. yoelii* was inoculated seven days prior to the inoculation of *P. vinckei*, virulence was much reduced, although the mixed infection still caused significantly more pathology and mortality than the constituent species in single infections. Similarly, when inoculated into mice contemporaneously with *P. vinckei*, *P. yoelii* suffered a reduction in cumulative parasite density throughout the infection (a proxy measurement of parasite fitness), whereas *P. vinckei* was unaffected. When *P. yoelii* was introduced to mice a week before *P. vinckei*, the opposite trend was observed, with little reduction in the cumulative parasite density of *P. yoelii*, but a significant reduction in that of *P. vinckei*. We show, therefore, that it is not just the phenotypes of the constituent species of mixed infections that affect pathology and parasite fitness in combination, but also the time at which each species infects the host.

It is possible that interactions between malaria parasite species in mixed infections may be modulated through the host immune response. Molineaux et al. (42) suggested that in mixed infections of *P. falciparum* and *P. malariae*, the immune response stimulated by rising *P. falciparum* parasitaemias can inhibit *P. malariae*, but that *P. falciparum* survives longer due to its more rapid growth rate. This immune-mediated antagonism (43) agrees with observations suggesting that *P. falciparum* could reduce the prevalence of *P. malariae* (44).

There is some evidence to suggest a degree of cross-protection between malaria parasite species due to species-transcending immunity (36, 45). We wondered whether the lack of ability to control the *P. vinckei* parasitaemia toward the end of the mixed infection of *P. vinckei* and *P. yoelii* may be due to the phenomenon of "original antigenic sin" (46) rendering the acquisition of antibodies specific to *P. vinckei* sub-optimal due to the larger quantity of *P. yoelii* antigen present in the early stages of the infection. To test this, we immunized mice through exposure to and subsequent cure of a *P. yoelii* infection, and then challenged with *P. vinckei*. Contrary to the expectations of the original antigenic sin hypothesis, we observed no effect on the severity of the *P. vinckei* infection in *P. yoelii*-exposed compared to non-exposed mice.

Infection with malaria parasites is known to detrimentally affect the fitness of the infected mosquito (47). Mixed species malaria parasite infections occur in nature (4, 48), and as mixed species parasite infections caused dramatically different disease outcomes in mice, we investigated whether mosquito fitness was also affected. We measured longevity and progeny production in groups of mosquitoes fed on single and mixed species infections. In contrast to the significant alterations in pathogenicity observed in mice, there appeared to be no fitness differences between mosquitoes carrying single or mixed species infections. Linked to this, we did not observe significantly increased oocyst numbers in mosquitoes infected with mixed species, when compared to the highest-oocyst producing single constituent species, suggesting there was no significant alteration in transmission-stage investment by the species in mixed stage infections (31).

We found that the transmissibility of *P. vinckei* and *P. chabaudi* to mosquitoes was reduced in the presence of *P. yoelii* in co-infections compared to single infections. This was reflected in the lower number of oocysts of these two species in mosquitoes that had fed on mixed species infections also containing *P. yoelii* compared to those that had fed on single infections. Most significantly, transmission of *P. chabaudi* to mosquitoes was blocked completely by the presence of *P. yoelii* on day 5 of the infection. There were no reductions, however, in the numbers of *P. yoelii* oocysts. Of the three species, *P. yoelii* produces significantly higher oocyst burdens in mosquitoes than either *P. chabaudi* or *P. vinckei*, a phenomenon linked to the former species having much higher gametocyte production during the early stages of infection. One possible mechanism that may account for this observation involves gamete incompatibility; we propose that given the fact that in a mixed species infection containing *P. yoelii*, there will be significantly more *P. yoelii* microgametes than of the other species. If *P. yoelii* microgametes can recognize and attempt to fertilize the macrogametes of the second species, then a large proportion of these macrogametes will be rendered non-productive (assuming hybrids are non-viable) (49). Consistent with this theory is the fact that both *P. chabaudi* and *P. vinckei* also produce fewer oocysts when in mixed infection with each other, but much less so than when mixed with *P. yoelii*, reflecting, perhaps, the more even numbers of gametocytes produced by these two species.

A limitation of these experiments is that mosquitoes were allowed to feed on mice at only two time points, days 3 and 5 post-inoculation, rather than throughout the course of the infection. These two time points were chosen as they represent the days of maximum transmissibility of *P. yoelii* (day 3), *P. chabaudi,* and *P. vinckei* (day 5). However, these results offer only a snapshot of transmission success on these particular days, and it is possible that different outcomes would have been observed at different time-points throughout the infection.

The experiments described here were conducted using multiple strains of three species of rodent malaria parasites in 6-week old, female CBA mice. If the same experiments were to be conducted with different parasite strains and species, and in different host strains, then different outcomes might be expected. The disease progression of rodent malaria parasites is dramatically affected by mouse host strain, sex, and age (50), and it is likely that the interactions between species in mixed infections would be similarly affected. It should also be remembered that *Mus musculus* is not the natural host of the rodent malaria parasites, and neither is *Anopheles stephensi* its natural vector. It is likely that the interactions between the species tested here would result in different outcomes in their natural hosts. Furthermore, blood stage infections in mice were initiated by intravenous inoculation of infected blood, and not by the

more natural route of sporozoite inoculation, another factor that may have a significant impact on the outcome of mixed species infections.

An illustration of the degree to which different outcomes may be observed in different experimental systems is given by the comparison of our results with those of Snounou et al. (34). Whereas, we observed dramatic increases in host mortality in mixed species infections containing a normocyte specialist and *P. yoelii*, Snounou et al. observed the opposite effect; with less mortality in *P. vinckei* + *P yoelii* and *P. chabaudi* + *P. yoelii* mixed species infections than in single infections of the normocyte-invaders (34). Similarly, although Ramiro et al. reported increased virulence in mixed infections in a similar manner to our observations, the mechanism for this outcome appears to differ; in our case facilitation of the normocyte invading species led to increased parasitaemia, whereas Ramiro et al. report increased growth of the reticulocyte invading species (27). In both these examples, there were differences between experiments in crucial parameters such as the sex, strain and age of the host mice used, the particular strains of malaria parasites, their passage histories, and routes of inoculation; all factors known to influence the outcome of experimental infections.

We cannot, then, make any predictions about the possible effects of mixed species human infections based on the results presented here, except to expect that there would be interactions between parasite species within the host, and that these would impact on disease outcomes.

As the world moves toward reducing the burden of malaria, the importance of mixed-species malaria infections will rise. Diagnostic techniques with improved sensitivities are revealing a greater prevalence of *P. ovale* and *P. malariae* in *P. falciparum* endemic areas than previously thought (7). Intervention strategies such as anti-vector programs, and the development and employment of new drugs and vaccine will often be more effective against one species of parasite than they will against others (51, 52). In regions where *P. falciparum* prevalence is decreasing, the prevalence of non-falciparum malaria parasite species often becomes more apparent, highlighting the importance of mixed-species infections. There is a need, therefore, to better understand how the interactions between malaria parasites species infecting the same host can impact disease progression and parasite fitness.

The experiments described here show that the disease outcomes of mixed vs. single species infections can differ and may be influenced by the phenotypic characteristics of the constituent species and the order in which they infect the host.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Animal experimentation was performed in strict accordance with the Japanese Humane Treatment and Management of Animals Law (law No. 105, 1973; modified 2006), and according to the Regulations on Animal Experimentation at Nagasaki University, Japan. All procedures were approved by the Institutional Animal Research Committee of Nagasaki University.

AUTHOR CONTRIBUTIONS

RC and JT designed, performed, and analyzed the experiments. RC, JT, TT, and JC 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.2019.03072/full#supplementary-material

Supporting Table 1 | Details of statistical treatments used in the study.

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Conflict of Interest: The 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.

frontiers in Immunology

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Monoclonal IgM Antibodies Targeting *Candida albicans* Hyr1 Provide Cross-Kingdom Protection Against Gram-Negative Bacteria

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Recent years have seen an unprecedented rise in the incidence of multidrug-resistant (MDR) Gram-negative bacteria (GNBs) such as *Acinetobacter* and *Klebsiella* species. In view of the shortage of novel drugs in the pipeline, alternative strategies to prevent, and treat infections by GNBs are urgently needed. Previously, we have reported that the *Candida albicans* hypha-regulated protein Hyr1 shares striking three-dimensional structural homology with cell surface proteins of *Acinetobacter baumannii*. Moreover, active vaccination with rHyr1p-N or passive immunization with anti-Hyr1p polyclonal antibody protects mice from *Acinetobacter* infection. In the present study, we use molecular modeling to guide design of monoclonal antibodies (mAbs) generated against Hyr1p and show them to bind to priority surface antigens of *Acinetobacter* and *Klebsiella pneumoniae*. The anti-Hyr1 mAbs block damage to primary endothelial cells induced by the bacteria and protect mice from lethal pulmonary infections mediated by *A. baumannii* or *K. pneumoniae*. Our current studies emphasize the potential of harnessing Hyr1p mAbs as a cross-kingdom immunotherapeutic strategy against MDR GNBs.

Keywords: monoclonal antibodies, *Candida* Hyr1, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, passive vaccine, molecular modeling, cross-kingdom immunotherapy

INTRODUCTION

Infections caused by multidrug-resistant organisms (MDROs) pose increasing therapeutic challenges. In the past decade, *Acinetobacter baumannii* has emerged as one of the most common MDROs in hospital-acquired infections, causing a range of diseases from pneumonia to sepsis or wound infections (1–6). Of great concern is that 40–70% of *A. baumannii* isolates are now extensively drug resistant (XDR; i.e., resistant to all antibiotics except colistin or tigecycline), reflecting a >15-fold increase since 2000 (1, 6–8). Likewise, the Enterobacteriaceae organism *Klebsiella pneumoniae* causes high rates of morbidity and mortality in critically ill, hospitalized patients. In recent years, strains of *K. pneumoniae* have exhibited resistance to almost all classes

of antibacterial drugs, including carbapenems (9–11). Together, *Acinetobacter* and carbapenem-resistant *K. pneumoniae* (KPC) have been prioritized by the U.S. Centers for Disease Control and Prevention (CDC) as two of the top "serious threat level pathogens" owing to resistance, failure of the current standard of treatment, and high mortality rates. Amplifying these concerns, the existing drug development pipeline against these pathogens is sparse, and it is almost certain that these organisms will develop resistance to any future approved antibiotics. Hence, novel strategies to prevent and treat life-threatening infections caused by these and related MDRO pathogens are urgently needed.

We previously developed innovative computational molecular modeling and bioinformatics strategies to discover novel vaccine and immunotherapy candidates targeting more than one high-priority pathogen. The application of this methodology has been used to successfully discover and develop novel cross-kingdom vaccines (12). Among other advances, this discovery strategy culminated in the identification of *Candida albicans* Hyr1p, a hypha-regulated cell surface protein. Although Hyr1p is strictly expressed on *C. albicans* hyphae, it has no effect on the fungus germination and subsequent hyphal formation (13). However, we have shown that Hyr1p contributes to *C. albicans* virulence by resisting phagocyte killing (a major host defense mechanism against candidiasis) through a mechanism that is yet to be identified (14). Indeed, mice vaccinated with Hyr1p are protected from *C. albicans* infections (14, 15). Recently, we found that the Hyr1p shares striking three-dimensional (3-D) structural and epitope homologies with antigens present on the Gram-negative bacterium (GNB) *A. baumannii*, including with the putative hemagglutinin/hemolysin protein FhaB, outer membrane protein class A (OmpA), and a number of siderophore-binding proteins (16). All these putative cross-reactive antigens are known contributors to bacterial virulence. Specifically, FhaB and OmpA help in bacterial adhesion and biofilm formation (17–19). Also, OmpA plays a role in conferring multidrug resistance of *A. baumannii* to antibiotics (20). Finally, the *A. baumannii* siderophore acinetobactin was shown to be required for bacterial infection by acquiring iron from the host (21), therefore implicating siderophore receptors in the virulence of the bacterium. Polyclonal antibodies (pAbs) raised against peptides derived from the Hyr1p N-terminus blocked *A. baumannii*-mediated lung epithelial cell damage and killed the bacterium *in vitro* (16). Importantly, anti-Hyr1p pAbs completely protected mice from *A. baumannii* infections. These results provided compelling proof of concept for targeting Hyr1p for developing immunotherapies against GNBs and laid a groundwork for generation and evaluation of the efficacy of anti-Hyr1p monoclonal antibodies (mAbs) targeting MDR GNBs.

In the current study, we generated mAbs against peptide #5 of Hyr1 and affirm that these mAbs not only recognize different clinical isolates of *A. baumannii* but also bind to drug-resistant *K. pneumoniae*. We further demonstrate the efficacy of these targeted mAbs in blocking bacterial-mediated host cell damage and in protecting mice against lethal pulmonary infection by both MDR bacteria. Given that there are currently no immunotherapies against GNBs and the alarming rate at which MDROs are increasing as a global threat to public health, active, or passive vaccination strategies using vaccines or mAbs, respectively, are now highly attractive immunotherapeutic modalities to prevent or treat these refractory infections either as standalone or antibiotic-adjunctive therapies.

RESULTS

Hyr1p Is Structurally Homologous to Target Surface Antigens of *Klebsiella pneumoniae*

Our previous studies involving complimentary homology and energy-based modeling algorithms identified structural domains conserved between Hyr1p and the GNB *Acinetobacter baumannii* (16). We questioned if other GNBs could similarly share conserved physiochemical structural domains. Of great relevance, we identified strong homology between Hyr1p and filamentous hemagglutinin B (FhaB) of *Klebsiella pneumoniae* (**Figures 1A–D**). This highly conserved homology was reflected at the level of amino acid sequences in a shared target motif (**Figures 1A–B**), relative structural integration of this motif in the larger holoproteins (**Figure 1C**), and overall 3-D homology of the two proteins (**Figure 1D**). Furthermore, our modeling studies revealed four other proteins in *K. pneumoniae* that displayed conserved 3-D homology with Hyr1p: OmpA, transporter of nutrients B (TonB), fimbrial protein (Fmp), and the biopolymer export protein D (ExbD). Following energy minimization and hydrogen-bond optimization to yield 3-D structure models, these proteins were aligned further with a 14-amino-acid peptide of Hyr1p (LKNAVTYDGPVPNN; also called peptide #5)–a highly antigenic, surface-exposed domain of the protein, for which anti-peptide pAbs were shown to protect against murine *A. baumannii* infection (16). This was done to localize specific homology sites hypothesized to confer protective efficacy (**Figures 1A–D**). Modeling data demonstrated that the identified 3-D structures corresponded with a conserved sequence region within each target antigen (**Figure 1E**). Based on strong efficacy seen in cross-kingdom immunization studies of prior modeling-predicted antigens (e.g., Hyr1p vs. *A. baumannii*) (16), the current model predictions were interpreted as supporting confidence in cross-protective efficacy against *K. pneumoniae.*

Anti-Hyr1 Monoclonal Antibodies Bind to Gram-Negative Bacteria

We previously reported that pAbs raised against Hyr1 peptide #5 blocked virulence functions of *A. baumannii in vitro* and completely protected diabetic and neutropenic mice from *Acinetobacter* bacteremia and pulmonary infection, respectively (16). Encouraged by these results, and to enhance the therapeutic potential of such antibodies, we developed mAbs against the same surface-exposed and immunodominant peptide (peptide #5). These mAbs (all IgM isotypes) were tested for their abilities to bind to *Candida albicans* as well as the GNB *A. baumannii* or *K. pneumoniae.* Four individual fluorescein isothiocyanate (FITC)-labeled mAb clones (H1, H2, H3, and H4; 100 μg/ml) were tested against three prototypic MDR GNB strains, including

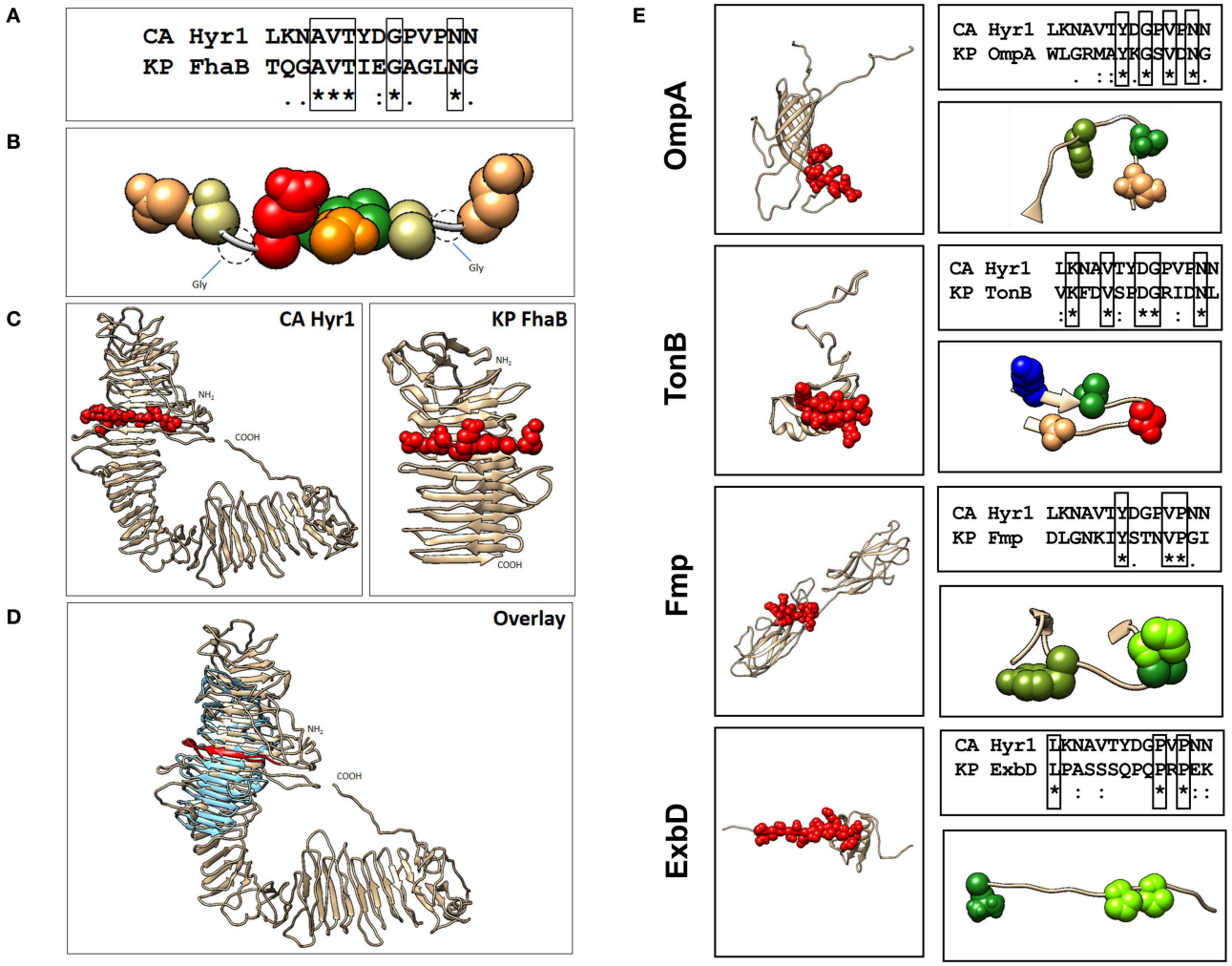

FIGURE 1 | Localization of Hyr1 peptide #5 and putative cross-reactive epitopes in modeled *Klebsiella pneumoniae* target sequences. **(A)** Sequence alignments between Hyr1 peptide #5 and putative cross-reactive motifs within *K. pneumoniae* FhaB sequence; identical residues are boxed. **(B)** van der Waals space-filling models illustrating conservation of amino acid physicochemistry in the highly homologous motifs; coloration is a modified RasMol schema (Gly, Ala–cream; Asn, Gln–khaki; Thr–orange; Val–green; Asp–red). **(C)** Comparative models of *Candida albicans* Hyr1 and *K. pneumoniae* FhaB showing homologous cross-reactive domains in red van der Waals space-filling spheres within the homologous domains of the two proteins. **(D)** Superimposition overlay of the homologous regions of Hyr1 and FhaB showing strong 3-D homology in antiparallel β-sheet facets and overall structures in which the conserved target motifs exist. Peptide #5 is shown in red as van der Waals space-filling spheres in the comparative Hyr1 and FhaB models. **(E)** Individual models for four targeted cross-reactive antigens of *K. pneumoniae* (OmpA, TonB, Fmp, and ExbD) are shown with domains homologous to Hyr1 peptide #5 in red. Sequence alignments between Hyr1 peptide #5 and cross-reactive target motifs with identical residues are boxed; also shown are domains showing identical and/or physicochemically conserved residues as space-filling spheres; coloration is a modified RasMol schema (Gly, Ala–cream; Asn, Gln–khaki; Thr, Ser–orange; Val, Ile, Leu, Met, Cys–green; Trp, Tyr, Phe–olive green; Asp, Glu–red; Arg, Lys–blue; His–sky blue; Pro–chartreuse).

A. baumannii (HUMC-1, XDR clinical isolate); *K. pneumoniae*-RM (KPC-RM, carbapenem-resistant clinical isolate); and *K. pneumoniae*-QR (KP-QR, MDR strain sensitive to carbapenem). The extent of mAb binding to each of the pathogen surfaces, as compared with isotype-matched non-specific control antibodies, was then quantified by flow cytometry. Of the four mAb clones tested, compared with the isotype-matched control IgM, H3, and H4 displayed the highest levels of binding to all GNBs, with at least 10–300-fold increases (**Figure 2A**). Binding potential was also visualized by a shift in the peaks of the anti-Hyr1p IgM binding vs. the isotype-matched control antibodies (**Figure 2B**). The right shift in the peaks of individual mAb also correlated with their respective increase in mean fluorescence of the cells.

Next, we compared the relative binding ability of the mAb clones to recognize the distinct GNBs. Clone H3 bound to all three organisms even at low mAb concentrations. Specifically, 30 μg/ml of H3 mAb bound 82, 75, and 90% of *A. baumannii* HUMC-1, KP-QR, and KPC-RM cells, respectively, whereas the isotype-matched control did not bind to any of the bacterial cells (<1%). The binding of the H3 mAbs to either *A. baumannii* or *K. pneumoniae* was maintained even at a very low concentration of 300 ng/ml, demonstrating 4–10-fold increase over the binding

A

Binding Antibody		Mean fluorescence intensity		
		HUMC-1	KP-QR	KPC-RM
☐	IgM Isotype	287	83	989
☐	H1	15,902	5,605	6,393
☐	H2	12,404	3,968	6,644
☐	H3	54,616	10,757	8,227
☐	H4	31,676	14,483	9,208

B

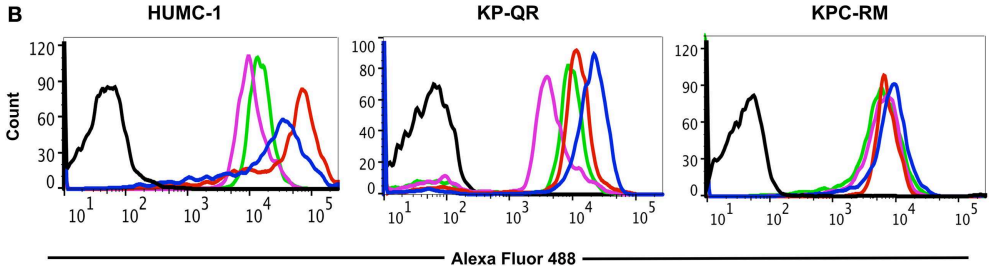

FIGURE 2 | Binding of monoclonal antibodies (mAbs) targeting Hyr1 peptide #5 to Gram-negative bacteria (GNBs). MAb clones (and isotype-matched control) IgM were evaluated for binding to *Acinetobacter baumannii* (HUMC-1), *Klebsiella pneumoniae*-QR (KP-QR), and *K. pneumoniae*-RM (KP-RM) at a concentration of 100 μg/ml. The extent of binding was quantified by flow cytometry after staining the bound antibodies with Alexa 488-conjugated secondary antibody. Data were represented as mean fluorescence intensity of the Ab-bound bacteria **(A)**. The degree of binding was also visualized by a shift in the peaks in the anti-Hyr1 IgM binding conditions vs. the control antibodies **(B)**.

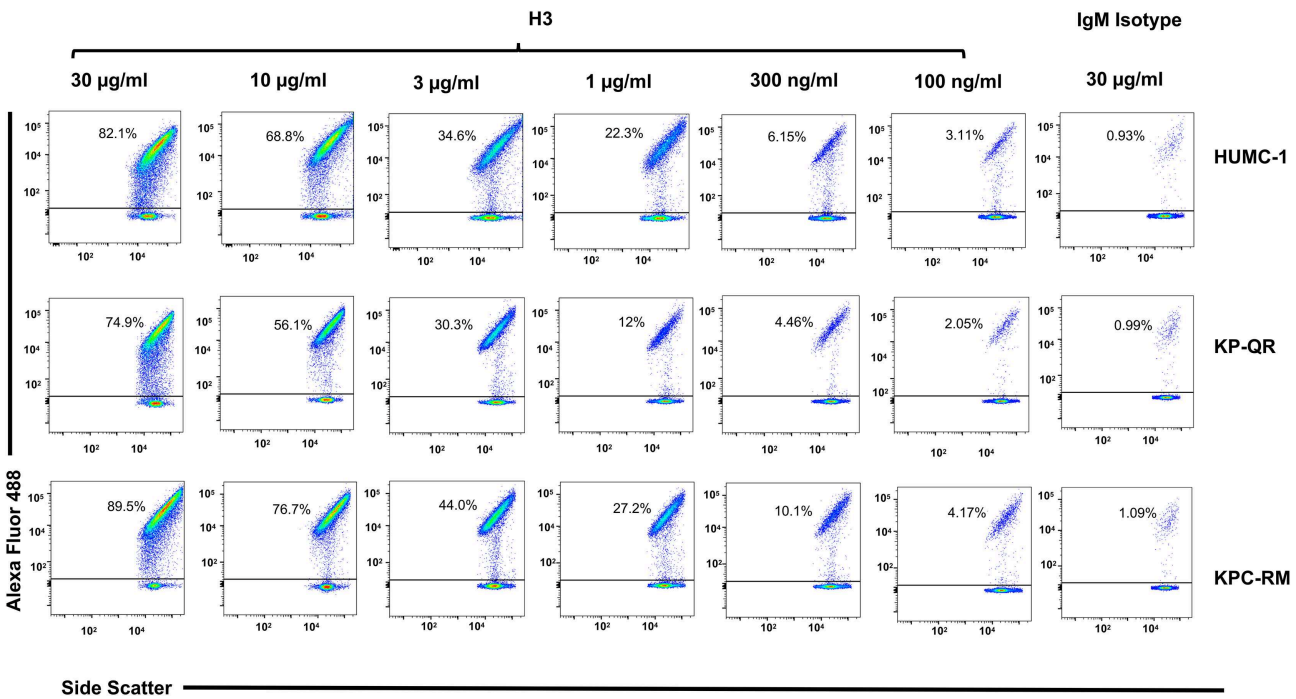

FIGURE 3 | Monoclonal antibody (mAb) clone H3 targeting Hyr1 peptide #5 binds Gram-negative bacteria (GNBs) in a dose-dependent manner. MAb clone H3 (and IgM isotype control) were evaluated for binding to HUMC-1, KP-QR, and KP-RM at concentrations ranging from 30 to 0.1 μg/ml. The extent of binding was quantified by flow cytometry after staining the antibodies with Alexa 488-conjugated secondary antibody. Data were represented in a scatter plot highlighting the percentage of bacteria that were bound by the Abs.

ability of the isotype-matched control IgM (**Figure 3**). Similarly, the H4 clone bound to each of the GNBs at 30 μg/ml of concentration (data not shown). We further evaluated the binding of the two clones H3 and H4 against other drug-resistant clinical isolates of *A. baumannii* and *K. pneumoniae* (KPC). The mAbs bound HUMC-6, HUMC-12, KPC-6, and KPC-8

at significantly higher capacity as than does the control IgM (**Supplementary Figure 1**). These results indicate that binding of mAbs to the surface of the target GNBs is not isolate specific, supporting our consensus epitope hypothesis.

Monoclonal Antibodies Protect Host Cells From Damage by Gram-Negative Bacteria

Our previous studies demonstrated that anti-Hyr1p pAbs not only bound to *A. baumannii* but also inhibited the ability of bacterium to interact with and damage mammalian cells (16). Thus, we hypothesized that the mAbs would similarly block damage of host cells caused by these GNBs. Concordant with this hypothesis, mAb clones H3 and H4 at 15 or 30 μg/ml blocked the ability of *A. baumannii* (HUMC-1) and *K. pneumoniae* (KP-QR or KPC-RM) to damage A549 lung alveolar epithelial cells. Specifically, both mAbs showed a dose–response inhibition of GNB-mediated A549 cell damage with the 15 μg/ml of dose resulting in 40–90% inhibition and the 30 μg/ml dose causing ∼70–100% inhibition (**Figure 4A**). The two mAbs also protected A549 cells from damage by other clinical isolates of GNBs such as HUMC-6 and KPC-8 (**Supplementary Figure 2**). Consistent with these results, both mAbs at 15 μg/ml resulted in ∼40–70% inhibition of *A. baumannii* HUMC-1- or KP-QR-mediated damage to primary human umbilical vein endothelial cells (HUVECs). However, it took a higher mAb concentration, 30 μg/ml, to protect HUVECs from KPC-RM (**Figure 4B**). Overall, these results show that mAbs raised against Hyr1 peptide #5 bind to MDR *A. baumannii* and *K. pneumoniae* strains and mitigate the ability of these bacteria to damage host cells *in vitro*.

Anti-Hyr1 Monoclonal Antibodies Protect Mice From Pulmonary Infection Caused by *Acinetobacter baumannii* or *Klebsiella pneumoniae*

We tested the ability of the mAbs, given their efficacy in reducing GNB-induced host cell damage *in vitro*, to protect mice from GNB infection. Pneumonia is a life-threatening manifestation of the disease caused by both *A. baumannii* and *K. pneumoniae* (22–25). Thus, we evaluated H3 and H4 for their ability to protect against such infections caused by *A. baumannii* HUMC-1. Although benign in immunocompetent individuals, *A. baumannii* can cause life-threatening pneumonia in immunosuppressed hospitalized patients (25). Thus, we additionally evaluated the efficacy of mAb therapy in a neutropenic mouse model infected with HUMC-1 *via* inhalation. The mAbs were administered intraperitoneally (i.p.) at a dose of 30 μg/mouse, in established infection on Days +1 and +4 relative to infection. Placebo mice were treated in an identical regimen with an isotype-matched control IgM. Treatment with mAb H4 yielded a high (70%) overall survival, vs. 20% overall survival for control IgM treatment ($P < 0.06$). Impressively, complete protection (100% survival) was conferred in mice receiving H3 mAb treatment, $P < 0.001$ (**Figure 5A**). Surviving mice appeared healthy on Day +21 post infection, when the experiment was terminated.

We next evaluated the efficacy of mAbs in a similar mouse model of *K. pneumoniae* pulmonary infection. Our *in vivo* optimization studies showed that KP-QR exhibits pronounced lethality even in healthy immune competent mice (**Supplementary Figure 3A**), whereas KPC-RM is avirulent despite high inocula used for infection (**Supplementary Figure 3B**). Thus, we evaluated the protective effect of mAbs against the KP-QR-mediated pneumonia in mice. Immunocompetent mice were infected intratracheally with KP-QR and treated twice as above with either the H3 or H4 mAbs, or isotype-matched control IgM. Almost 60% of mice treated with H4 survived the otherwise lethal challenge by KP-QR ($P < 0.05$). Consistent with protection against host cell damage, even greater efficacy was observed with mAb H3, which exhibited protection trending to 80% survival vs. 20% survival in mice treated with isotype-matched IgM ($P < 0.05$) (**Figure 5B**). Surviving mice appeared healthy at 21 days post infection at the experimental endpoint.

We also performed studies to assess the effect of the mAb treatment on the bacterial burden in lung tissues, along with survival efficacy. Mice were infected as above and treated with H3 mAb once at 6 h post infection for KP-QR and twice (6 h and a repeat dose on Day +3 post infection) for *A. baumannii* HUMC-1. Mice were sacrificed on Day +2 for KP-QR and Day +4 for HUMC-1, and the lungs were harvested for bacterial burden enumeration by quantitative culture. Corroborating the survival data and in comparison with treatment with isotype-matched IgM control, H3 mAb treatment resulted in 1.5- or 3-log reductions in lung bacterial burden of HUMC-1 ($P < 0.01$) or KP-QR ($P < 0.001$), respectively (**Figure 5C**).

Together, these results demonstrate that therapeutic mAbs derived from innovative methods to exploiting cross-kingdom epitope homology exhibit striking efficacy in life-threating GNB infection. These results emphasize the strong proof-of-concept translational potential to develop such agents as novel therapeutic modalities for prevention or treatment of infections due to MDR GNBs in immunocompetent as well as immunosuppressed or immunodeficient patients.

DISCUSSION

Phylogenetically diverse pathogens may exploit common host settings and rely on convergent virulence strategies (e.g., cell adhesion, invasion, and injury). Indeed, the fungus *Candida albicans* and certain GNBs, such as *Acinetobacter baumannii* and *Klebsiella pneumoniae*, infect similar immunocompromised, burn, and surgical wound patients in intensive care units (ICUs) or otherwise hospitalized (7, 19, 26). Interestingly, *Candida* species colonization among ICU patients have been identified as an independent risk factor for development of *A. baumannii* ventilator-associated pneumonia (27). Similarly, *Candida* and *Klebsiella* are the most frequent pathogens of the respiratory tract of patients with chronic obstructive pulmonary disease (COPD) (28, 29). Independent of such an association, *Candida* and GNBs individually cause healthcare-associated infections, often leading to significant morbidity and mortality. As a group, GNBs in

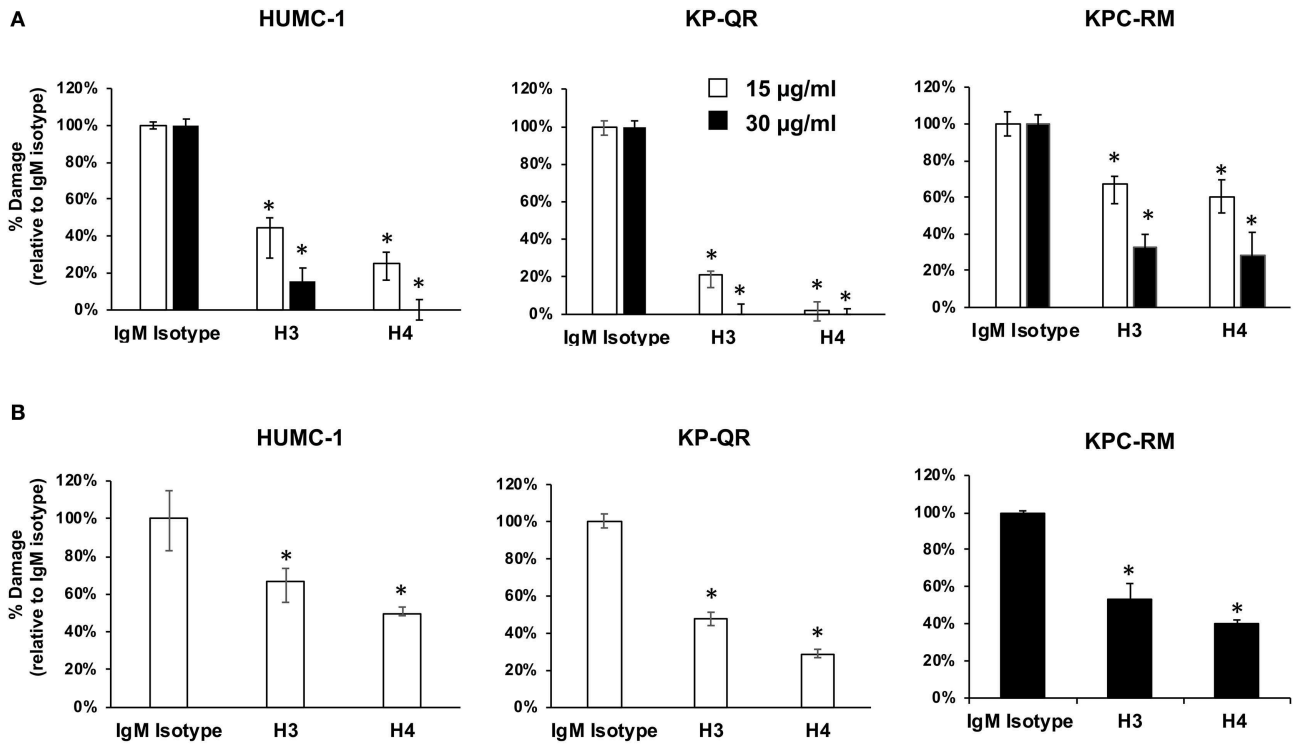

FIGURE 4 | Monoclonal antibodies (mAbs) targeting Hyr1 peptide #5 prevents HUMC-1-, KP-QR-, and KP-RM-induced A549 lung alveolar epithelial cells and primary human umbilical vein endothelial cells (HUVECs) damage. HUMC-1-, KP-QR-, and KPC-RM-induced A549 cell injury in the presence of 15 or 30 μg/ml of an isotype-matched IgM, mAb H3, or mAb H4 **(A)**. Damage to HUVECs by HUMC-1 and KP-QR (in the presence of 15 μg/ml of H3 and H4), also KPC-RM (30 μg/ml of the mAbs) **(B)**. Cell damage was determined by ^{51}Cr-release assay after 48 and 24 h for HUMC-1 and KP, respectively. Percentage damage was normalized to IgM isotype-matched control after subtracting spontaneous cell damage. *$P < 0.001$ vs. control IgM. $N = 12$ per group from three independent experiments.

particular, including *A. baumannii* and *K. pneumoniae*, have evolved into MDR pathogens that cause infections that are often incurable (30). Hence, novel approaches that address antibiotic resistance and leverage or amplify immune function represent highly logical strategies to combat this resistance crisis.

Our group has developed advanced computational, molecular modeling, and bioinformatics strategies to discover novel vaccine antigen candidates that leverage the concept of convergent immunity to target more than one high-priority human pathogen (16, 31, 32). This strategy, also known as unnatural or heterologous immunity, has been previously applied in the development of viral and bacterial vaccines in which an antigen protects against another pathogen from the same or from a different kingdom (12, 25). We have previously validated this approach by demonstrating cross-kingdom immuno-protection against *C. albicans* and *Staphylococcus aureus*, in which the *C. albicans* cell surface adhesin/invasion proteins [agglutinin-like sequence (Als) family of proteins] share epitope and functional homology with MSCRAMMs of *S. aureus* (e.g., clumping factor A) (33). A recombinant form of N-terminus of the Als3p (rAls3p-N) elicits robust T- and B-cell responses and protects mice from both *Candida* and methicillin-resistant *S. aureus* (MRSA) infections (32, 34–39). Most recently, we reported that a distinct hyphal cell surface protein of *C. albicans*, Hyr1p, has epitope homologies with candidate antigens of the MDR GNB *A. baumannii* (16). Indeed, with the use of different mouse models, active or passive immunization (with pAbs) targeting either Als3p or Hyr1p protected mice from *S. aureus* or *A. baumannii* infections, respectively (16, 32, 34). In particular, antibodies against one specific surface-exposed and highly antigenic 15-mer peptide of Hyr1 (peptide #5) offered the highest protection to host cells from *A. baumannii* both *in vitro* and *in vivo* (16).

Homology and energy-based modeling was conducted to compare the overall and target motif-specific physicochemical features of Hyr1 protein with candidate *K. pneumoniae* target antigens. These methods predicted Hyr1p to share 3-D and sequence conservation with a number of proteins expressed on the *K. pneumoniae* surface. In addition to FhaB, significant homologies were observed between the Hyr1 peptide #5 domain that induced highly protective antisera, and OmpA, TonB, Fmp, and ExbD common to *K. pneumoniae* and other high-priority GNBs. Encouraged by the potential of the pAbs, and to further the clinical relevance of our studies, we generated mAbs against the highly antigenic peptide #5 of the Hyr1p. Similar to pAbs, the current results demonstrate that the mAbs blocked the MDR *A. baumannii*- or *K. pneumoniae*-mediated host cell damage and protected mice from otherwise lethal pulmonary infections caused by these pathogens. Initial functional assays revealed that four different mAb clones (H1–H4) recognized the two genera of bacteria for *in vitro* binding at low concentrations

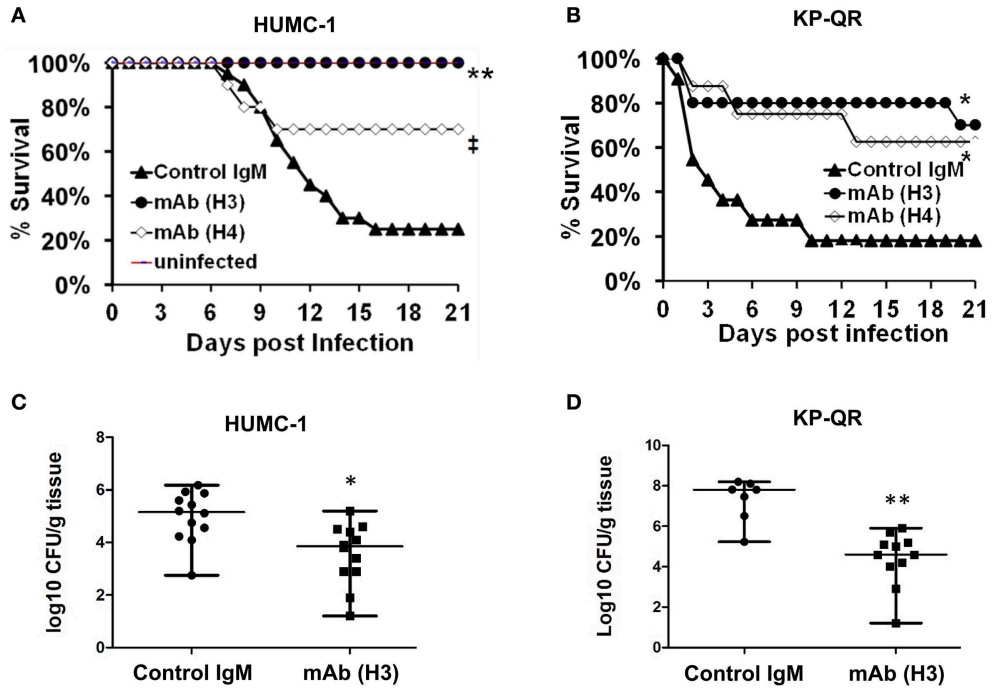

FIGURE 5 | Monoclonal antibody (mAb) clones H3 and H4, targeting Hyr1 peptide #5, protect mice from HUMC-1- or KP-QR- induced pneumonia. Immunosuppressed CD-1 male mice ($n = 10$/group from two experiments) were infected with HUMC-1 *via* inhalation [average 5×10^{10} colony-forming units (CFU)] **(A)**. Immunocompetent mice ($n = 10$/group from two experiments) were infected intratracheally with KP-QR (average 3.4×10^7 CFU) **(B)**. Intraperitoneal (i.p.) treatment with mAb H3, H4, or isotype-matched control IgM started 24 h and repeated at 96 h post infection (30 μg/mouse each dose). *$P < 0.05$, $P = 0.06$, and **$P < 0.001$ vs. control IgM by log-rank test. For CFU measurement, H3 and control IgM were administered 6 h and 3 days post infection, and lungs harvested from mice at Day +4 (for HUMC-1) **(C)** and at Day +2 (for KP-QR) post infection **(D)**.

of the antibodies. This targeting propensity was extended also to include several MDR clinical isolates of *A. baumannii* and *K. pneumoniae*.

The ability of the mAbs (H3 and H4) to block GNB-mediated damage of host cells was more pronounced in *A. baumannii* HUMC-1, *A. baumannii* HUMC-6, and *K. pneumoniae* KP-QR than in KPC-RM or KPC-8. Virulence factors, including capsule, lipopolysaccharide, fimbriae, and siderophores, have been identified as important for the virulence and/or resistance of KP strains to antibiotics (40). Thus, the resistance of KPC strains KPC-RM or KPC-8 to mAbs could conceivably be due to the difference in the exostructure of these organisms [e.g., differences in lipopolysaccharide (LPS) hindrance and reported production of a larger repertoire of siderophores] (40). Consistent with this hypothesis, our recent findings emphasized the importance of anti-Hyr1 peptide #5 pAbs in blocking iron uptake, leading to killing of the GNB *A. baumannii* (16).

Importantly, the mAbs afforded nearly 70% protection from cellular damage by all GNBs tested, supporting their potential use as preventive or therapeutic modalities with a capacity to block virulence of GNBs. Our recent report using bioinformatics, homology, and energy-based modeling strategies established that *C. albicans* Hyr1p shares striking epitope homology to *A. baumannii* FhaB protein, and anti-peptide #5 pAb bound to FhaB as well as two other proteins on *A. baumannii* based on two-dimensional Western blotting assays (16). The other two *A. baumannii* proteins with considerable homology to Hyr1p included the OmpA, and a ferric siderophore outer membrane binding protein (TonB) (16). Not surprisingly, these three proteins are well conserved in *K. pneumoniae* displaying >60% sequence homology [protein sequence National Center for Biotechnology Information (NCBI) blast alignment] and even greater 3-D homology to their *Acinetobacter* counterparts. Whether these proteins have a significant role in virulence or nutrient uptake–and hence blocking their function would contribute to the killing mechanisms afforded by the mAbs–is the subject of ongoing research by our group.

Because the mAbs significantly blocked the capacity of GNBs to damage host cells *in vitro*, we evaluated their potential to protect against lethal pulmonary infections caused by two prototypic MDR GNBs. In a validated mouse model, mAb H4 afforded >60% survival to infection by KP-QR as well as HUMC-1, as compared with control IgM having a 20% survival rate. Moreover, the mAb H3 provided 80–100% survival protection to mice from either of these GNBs, similar to that conferred by pAb (16). The efficacy seen by both H3 and H4 mAbs is afforded at the low concentration of 30 μg/mouse. This low dose of the mAb is about 1.2 mg/kg, which is within the dosage range of 1–15 mg/kg of most mAbs approved for human use (41, 42). This efficacy suggests that the mAbs likely neutralize functions of specific targets on the bacteria and attenuate their ability to exert virulence mechanisms or to cause disease in the

host. This hypothesis is further supported by the observation that treatment with mAbs significantly mitigated dissemination of KP-QR and HUMC-1 to distal target organs within 2–4 days of treatment, vs. mice treated with control antibodies. These results provide compelling evidence of the robustness of the antibodies in abrogating pathogenesis, early in the onset of infection as well as in the setting of established infection.

In addition to specificity and safety, a key advantage of using mAbs as anti-infective therapy is their well-documented long half-life, which can exceed 21 days (43, 44). Because the patient population at risk of developing infections with *Acinetobacter*, *Klebsiella*, and *Candida* are well-defined, this property of mAbs may afford an extended protection during the time span of the greatest vulnerability. In turn, prevention of such infections would translate to reduced use of antibiotics and hence a reduced pressure for the emergence of drug resistance. For example, mAbs can be used to prophylax patients at risk of MDR GNBs. Another envisioned usage of these mAbs is their administration as adjunctive therapy with antibiotics. In this respect, we have demonstrated synergy of anti-peptide #5 pAb with imipenem or with colistin in killing *A. baumannii* at reduced minimum inhibitory concentration (MIC) of both antibiotics (16). Similarly, the anti-peptide #5 pAb synergistically acted with colistin in abrogating biofilm growth of *A. baumannii* (16).

In theory, one caveat for using novel Abs to treat infections caused by organisms known to develop antimicrobial resistance is the potential development of resistance to these Abs. However, cross-resistance between small molecule antimicrobials and antibacterial mAbs is unlikely because of the distinct therapeutic targets and pharmacological mechanisms that antibodies have as compared with traditional antimicrobials (45). In concept, other potential bacterial defense mechanisms could occur, such as synthesis of antibody-neutralizing proteins (e.g., protein A of *S. aureus*, which binds antibody Fc domain and prevent opsonophagocytosis (46)), or proteases to degrade the administered mAb (47, 48). However, none of the antibodies approved for treating infectious diseases [currently, there are only three Food and Drug Administration (FDA)-approved mAb to treat inhalational anthrax (49, 50) or *Clostridium difficile* (42) have encountered this issue, and development of resistance has not been reported.

In summary, we have demonstrated that mAbs raised against peptide #5 of Hyr1 target *A. baumannii* and *K. pneumoniae* and disrupt their ability to damage to host cells *in vitro*. More importantly, these mAbs protect mice from lethal pulmonary infections mediated by two high-priority GNBs. Thus, such mAbs have credible potential for development as prophylactic or adjunctive therapy to prevent or treat life-threatening infections in patients susceptible to MDR *A. baumannii* or *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are clinical isolates collected from Harbor-UCLA Medical Center (Torrance, CA). *Acinetobacter baumannii* strains HUMC-1 and HUMC-6 were separated from patients' sputum, and HUMC-12 was separated from a patient's wound and are XDR to all antibiotics, except colistin and tigecycline. *Klebsiella pneumoniae* strains were categorized into KPC or non-KPC isolates. The KPC-RM, KPC-6, and KPC-8 isolates resistant to carbapenem antibiotics possess *bla* KPC plasmid gene and separated from patients' sputum, whereas KP-QR is a non-KPC but multidrug-resistant isolate separated from a patient's sputum and resistant to gentamicin, kanamycin, and ampicillin/sulbactam antibiotics. All bacteria were cultured in tryptic soy broth (TSB) overnight at 37°C with shaking at 200 rpm. To obtain a log-phase bacterial suspension, overnight cultured bacteria were passaged in a fresh media (1:100) at 37°C with shaking for 3 h or until the cell concentration reached an OD_{600} of 0.5 ($\sim 2 \times 10^8$ cells/ml) for both *A. baumannii* and *K. pneumoniae* isolates. The bacteria were diluted to the desired concentration from this stock.

Computational Modeling of Structural Homology

Our previously validated Hyr1 model (16) was used as a template to seek structural homologs in *K. pneumoniae* having predicted epitopes for cross-kingdom immune protection. The Phyre 2.0 (51) and iTasser (52) platforms were used to generate homology models for Hyr1 and putative-related proteins. Results were scored based on 3-D threading homology and sequence relatedness and were integrated to identify conserved structural domains. As a confirmatory measure, additional stochastic modeling was carried out using the Quark server (53). Select regions of resulting comparative homologs were then subjected to 3-D alignment to identify areas of greatest homology using the Smith–Waterman (54) algorithm as implemented within Chimera (55). Sequence alignments to identify putative shared epitopes between Hyr1 and other proteins were carried out using CLUSTALW (56).

Generation of Monoclonal Antibodies

Thirty micrograms of rHyr1 peptide #5 (synthesized by ProMab Biotechnologies, Richmond, CA) in 1 mg/ml of alum was used to immunize Balb/c mice ($n = 10$). The mice were boosted two times every 2 weeks with the same antigen concentration. Two weeks after the last boost, antibody titer was determined by ELISA plates coated with rHyr1 peptide #5. The spleens were collected, and the splenocytes were fused with hypoxanthine-guanine phosphoribosyltransferase (HGPRT)-negative murine myeloma cells at ratio 5:1 by slowly adding polyethylene glycol (PEG) to the cells pellet followed by adding Protein Free Hybridoma Media (PFHM) (Gibco, 12040077) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Corning, 35-016-CV). The cells were spun and re-suspended in 20% FBS PFHM and then incubated in 24-well plate at 37°C with 5% CO_2 for 48 h. The media were replaced with hypoxanthine–aminopterin–thymidine (HAT) selection media for 8 days and then 20% FBS PFHM hypoxanthine-thymidine (HT) media for 2 weeks. The grown hybridoma clones were diluted by microdilution in microtiter plates to achieve one cell per well and propagated in 10% FBS PFHM. The supernatant from the grown clones was tested for anti-Hyr1 antibodies using ELISA. The selected positive and stable clones were cultured in PFHM

without FBS, and the cell numbers were adjusted to be 2×10^5/ml for optimum production of mAbs.

Detoxification and Purification of the Supernatant Containing the Monoclonal Antibodies

The supernatant containing the antibodies was concentrated using 100-kDa cutoff centrifugal concentrating tube (Amicon, UFC910024). HiTrap HP column (GE Healthcare, 17511001) was used to purify the concentrated mAbs and then was buffer exchanged with endotoxin-free Dulbecco phosphate-buffered saline (PBS) without calcium or magnesium (Gibco, 14190250). Endotoxin was tested using a chromogenic limulus amebocyte lysate assay (BioWhittaker Inc.), and all mAbs had low-range endotoxin level of <0.015 EU/ml. The isotype of the mAbs was identified using ELISA and confirmed by molecular weight using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Surface Staining and Binding Assay

Bacterial cells (5×10^6 cells) re-suspended in 2% FBS–PBS were incubated with anti-Hyr1 mAbs or isotype-matched IgM control (BD Biosciences) for 2 h at a range of concentrations (100–0.1 μg/ml). The bacterial cells were washed three times with cold 2% FBS–PBS. The bound anti-Hyr1 mAbs to the bacterial cells were detected with anti-mouse FITC-labeled secondary antibodies (Thermo Fisher Scientific). The unbound antibodies were washed three times with cold 2% FBS–PBS before the measurement of the fluorescent-stained bacterial cells using flow cytometry (Becton Dickinson FACSCalibur), where it is adjusted to detect up to 20,000 events per sample.

Cell Damage Assay

The *in vitro* ability of mAbs to protect either A549 cells or HUVECs from damage caused by direct contact with bacteria was measured using ^{51}Cr release assay, modified from previous method (57). Isolation of HUVECs was performed in the laboratory under a protocol approved by institutional review board (IRB). Because umbilical cords are collected without donor identifiers, our IRB considers them medical waste and not subject to informed consent.

Alveolar epithelial A549 cells and HUVECs were incubated overnight in 24-well plates with F-12K or Roswell Park Memorial Institute (RPMI1640) medium supplemented with 10% FBS, containing 1 μCi/well of $Na_2\ ^{51}CrO_4$ (ICN Biomedicals, Irvine, CA). The next day, unincorporated tracer was aspirated, and the wells were rinsed three times with warm Hanks' Balanced Salt Solution (HBSS). One milliliter of media containing HUMC-1 or KP-QR (pre-incubated with mAbs or IgM isotype control for 1 h on ice) was then added to host cells in each well at a multiplicity of infection (MOI) of 1:100 (host cells to bacteria), and the plate was incubated for 48 or 24 h, respectively, at 37°C in 5% CO_2. At the end of the incubation, all the media were gently aspirated from each well, after which the mammalian cells were lysed by the addition of 0.5 ml of 6 N NaOH. The lysed cells were aspirated, and the wells were rinsed twice with RadioWash (Atomic Products, Inc., Shirley, NY). These rinses were added to the lysed cells, and the ^{51}Cr radioactivity of the medium and the cell lysates was determined. Control wells containing media but no organisms were processed in parallel to measure the spontaneous release of ^{51}Cr. After corrections were made for the differences in the incorporation of ^{51}Cr in each well, the specific release of ^{51}Cr was calculated by the following formula: (experimental release–spontaneous release)/(total incorporation–spontaneous release).

Animal Models

Male CD-1 immunocompetent mice (4–6 weeks old) were used for *Klebsiella* (KP-QR) intratracheal infection or immunosuppressed mice infected with *A. baumannii* (HUMC-1) by an aerosolization chamber to induce pneumonia by inhalation. Mice were immunosuppressed by administrating cyclophosphamide (200 mg/kg) (i.p.) and cortisone acetate (250 mg/kg) (subcutaneous) on Days −2, +3, and +8 relative to infection as previously described Gebremariam et al. (57). A total of 30 μg/mouse of mAbs or isotype-matched control were administrated (i.p.) on Day +1 and on Day +4 post infection. Survival of mice served as an endpoint. For quantitative measurement of bacterial burden, mAbs were administered 6 h after infection, and a repeat dose was given on Day +3. Mice were euthanized on Day +4 for *A. baumannii* and on Day +2 for *K. pneumoniae*. Lungs were harvested aseptically and homogenized, and the bacterial burden was determined by quantitative culturing on tryptic soy agar plates.

Statistical Analysis

The percentage of cell damage and tissue bacterial burden was compared using non-parametric Mann–Whitney test. The log-rank test was used to determine the difference in survival studies. $P < 0.05$ was considered significant.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

All procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center (protocol number 20295), according to the National Institutes of Health (NIH) guidelines for animal housing and care. Moribund mice according to detailed and well-characterized criteria were euthanized by pentobarbital overdose, followed by cervical dislocation.

AUTHOR CONTRIBUTIONS

EY performed conceptualization, data curation, formal analysis, investigation, methodology, and writing–original draft. SA, TG, and LZ performed investigation and methodology. SS performed data analysis and manuscript revision. NY performed methodological and data interpretation. MY performed conceptualization, methodology, data interpretation, and manuscript revision. PU performed formal analysis,

investigation, methodology, and writing–original draft. AI performed conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, and manuscript revision.

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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.2020.00076/full#supplementary-material

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Conflict of Interest: MY and AI are founders and shareholders of NovaDigm Therapeutics, Inc., which is developing novel immunotherapies targeting priority pathogens.

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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Preferential HLA-B27 Allorecognition Displayed by Multiple Cross-Reactive Antiviral CD8$^+$ T Cell Receptors

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T cells provide essential immunosurveillance to combat and eliminate infection from pathogens, yet these cells can also induce unwanted immune responses via T cell receptor (TCR) cross-reactivity, also known as heterologous immunity. Indeed, pathogen-induced TCR cross-reactivity has shown to be a common, robust, and functionally potent mechanism that can trigger a spectrum of human immunopathologies associated with either transplant rejection, drug allergy, and autoimmunity. Here, we report that several virus-specific CD8$^+$ T cells directed against peptides derived from chronic viruses (EBV, CMV, and HIV-1) presented by high frequency HLA-A and -B allomorphs differentially cross-react toward HLA-B27 allotypes in a highly focused and hierarchical manner. Given the commonality of cross-reactive T cells and their potential contribution to adverse outcomes in allogeneic transplants, our study demonstrates that multiple antiviral T cells recognizing the same HLA allomorph could pose an extra layer of complexity for organ matching.

Keywords: cross-reactivity, CMV, EBV, HIV-1, HLA, T cells, TCR

INTRODUCTION

A hallmark of human antiviral T cells is their ability to recognize viral peptide antigen bound to a self-human leukocyte antigen (HLA) on the surface of infected cells. Whilst this recognition often displays exquisite specificity, it is not uncommon for some of these T cells to cross-react with closely related peptide-HLA (pHLA) complexes, such as a peptide from a different viral strain (1). Given that T cells are inherently cross-reactive, by nature of thymic selection (i.e., recognition of self) and their interaction with foreign antigen in the periphery, cross-strain reactivity is a beneficial property affording protection to mutant viral strains and preventing immune escape. More remarkably, some T cells are also capable of recognizing apparently distinct pHLA including non-self or allogeneic pHLA (2–4), self-pHLA that have undergone some form of perturbation resulting in an altered self-peptide repertoire (5), and self-pHLA expressed in different tissues

(6). These forms of heterologous immunity, otherwise known as T cell cross-reactivity, are not beneficial to the host and can lead to transplant rejection, drug hypersensitivity and autoimmunity, respectively. Moreover, these potentially hazardous T cell responses are the price paid to maintain immune potential to combat the vast array of pathogenic challenges during a lifetime. Hence, cross-reactivity is an intrinsic feature of T cells, necessitated by the limited availability of unique human T cell receptor (TCR) clonotypes ($<10^8$ distinct TCRs) to maintain immunity against tremendous pathogenic diversity ($>10^{15}$ pHLA combinations) (7).

Childhood exposure to common viruses results in the induction of a robust immune response that controls the infection and generates long lasting immune memory. A small proportion of some viruses (e.g., herpesviruses including Epstein-Barr virus [EBV] and cytomegalovirus [CMV]) are able to evade the immune response by entering into a latent state inside the host cells. In fact, for these common herpesviruses up to 90% of individuals maintain viral latency by adulthood (8). The persistence of a memory pool of T cells against the virus generally controls outbreaks of viral reactivation. Recurrent reactivation episodes maintain these memory T cells at high frequency, facilitating rapid deployment and activation. Virally triggered cross-reactive T cells have predominantly been explored in infections where there is a high likelihood of their relevance after transplantation. This is particularly so for EBV and CMV, which establish latency in the host following naturally acquired or vaccine-induced immunity. These viruses have been directly implicated as risk factors associated with allograft rejection and graft vs. host disease (8), with studies demonstrating that high frequencies of herpesvirus-derived cross-reactive T cells (up to 85%) or clones (up to 45%) co-recognize alternate HLA allotypes (3, 9–11). Whilst there is a high likelihood that cross-reactive T cells are involved in clinical rejection (11–13), this has yet to be formally proven.

Allo-HLA cross-reactivity by antiviral T cells has been reported across a variety of HLA class I (A and B loci) and II (DRB1 locus) restricted targets [reviewed in (14)]. In some instances, antiviral T cells derived from either the same or heterologous viruses are capable of recognizing an identical HLA allomorph. For instance, HLA-B*44:02 is cross-recognized by B*08:01-restricted LC13 cytotoxic T lymphocytes (CTL; EBV EBNA3A$_{325-333}$), B*35:08-restricted SB27 CTL (EBV BZLF1$_{52-64}$) and A*02:01-restricted 5101.1999.23 CTL (herpes simplex virus-2 VP13/14$_{289-298}$) (15–19). Given the commonality of cross-reactive T cells and their potential to contribute to adverse immune responses in allogeneic transplants we wanted to determine whether multiple antiviral CTLs recognizing the same HLA allomorphs would contribute an extra layer of complexity for organ matching. This study examines the extent of T cell cross-reactivity generated by three heterologous viruses (i.e., EBV, CMV, and human immunodeficiency virus-1 [HIV-1]) toward different HLA-B27 allotypes, which may have clinical implications for transplantation.

Abbreviations: HD, healthy donor; HLA, human leukocyte antigen; LTR, lung transplant recipient; PBMC, peripheral blood mononuclear cells; pHLA, peptide-HLA; TCR, T cell receptor.

MATERIALS AND METHODS

Study Participants and Peripheral Blood Mononuclear Cells Isolation

Participant HLA typing is shown in **Supplementary Table 1**. Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and cryopreserved at −196°C until required.

Virus-Specific CD8$^+$ T Cell Lines or Clones

EBV, CMV, or influenza A (IAV)-specific CD8$^+$ T cell lines were generated from chronically-infected individuals following *in vitro* expansion of PBMC stimulated with gamma-irradiated peptide-pulsed autologous cells (1 μM peptide, 3,000 Rads) at a 2:1 ratio in RF10 [composed of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mM MEM non-essential amino acid solution (Life Technologies), 100 mM HEPES (Life Techologies), 2 mM L-glutamine (Life Technologies), penicillin/streptomycin (Life Technologies), 50 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 10% heat-inactivated FCS (Sigma-Aldrich)] supplemented with 20 U/mL IL-2 (PeproTech, Rocky Hill, NJ) for 13 days at 37°C, 5% CO_2 as previously described (4, 11). Peptides for CMV: HLA-A*02:01-restricted pp65-derived NLVPMVATV (A2$_{NLV}$) epitope, EBV: HLA-B*07:02-restricted EBNA-3A-derived RPPIFIRRL (B7$_{RPP}$) epitope and IAV: HLA-A*02:01-restricted matrix protein-derived GILGFVFTL (A2$_{GIL}$) epitope. Virus-specific CD8$^+$ T cell clones from chronically-infected individuals were generated following single-cell sorting based on tetramer staining using the HLA-B*57:01-restricted TSTLQEQIGW (B57$_{TW10}$) epitope derived from HIV-1 Gag protein for A16 and 457 (20) or EBV: B7$_{RPP}$ epitope for HD9G6 (21), as previously described (2, 22, 23).

Antigen-Presenting Cells and HLA Cell Surface Expression

C1R transfected cells expressing different HLA-I molecules (HLA-A*02:01, -B*07:02, -B*57:01, -B*27:01 to -B*27:10) were used as antigen-presenting cells (APCs), maintained in RF10 with selection antibiotics [Geneticin G418 (0.4–0.5 mg/ml; Roche Diagnostics, Mannheim, Germany) or hygromycin B (0.3 mg/ml; Life Technologies, Carlsbad, CA)] as required (4, 24). Increased HLA-I expression [compared to C1R Parental, which has low levels of HLA-A and HLA-B expression and normal HLA-C (25)] was confirmed via flow cytometry by indirect staining with appropriate antibodies; anti-human pan HLA-I (W6/32 hybridoma; for C1R.A*02:01, C1R.B*07:02, C1R.B*57:01 shown in **Supplementary Figure 1A**), anti-human HLA-B7/27 (ME1 hybridoma; for C1R.B*27:01 to C1R.B*27:10 shown in **Supplementary Figure 1B**) and a secondary goat anti-mouse IgG phycoerythrin (PE) (1:200 dilution; Southern Biotech, Birmingham, AL). All hybridomas were produced in-house. Stained cells were acquired on LSRII flow cytometer [Becton

Dickinson (BD), San Jose, CA]. Flow cytometry data was analyzed using FlowJo software (TreeStar, Ashland, OR).

Specificity and Functionality of CD8$^+$ T Cell Lines or Clones

The specificity and activation of virus-specific CD8$^+$ T cells were assessed by anti-CD8 and tetramer ($A2_{NLV}$ or $B7_{RPP}$) co-staining, followed by intracellular staining (ICS) for functional Th1 cytokine production using flow cytometry (11, 26). Briefly, 2×10^5 day 13 T cells were stimulated with media (negative control), Dynabeads® Human T-Activator anti-CD3/CD28 (positive control; Life Technologies) or 1×10^5 APC (±1 μM peptide) for a total of 6 h at 37°C, 5% CO_2 with 10 μg/mL brefeldin A (Sigma-Aldrich) added for the last 4 h. T cells were phenotyped with anti-CD8 PerCP Cy5.5 or allophycocyanin (APC) (1:20 or 1:40 dilution, clone SK1, BD Biosciences, San Jose, CA), HLA-$A2_{NLV}$ or HLA-$B7_{RPP}$ tetramer (conjugated to either PE or APC) and LIVE/DEAD fixable aqua stain (1:750 dilution, Thermo Fisher Scientific, Waltham, MA). T cells were then fixed in 1% paraformaldehyde (ProSciTech, Kirwan, Queensland, Australia), permeabilized in 0.3% saponin (Sigma-Aldrich) containing anti-IFNγ PE-Cy7 (1:250 dilution, clone B27, BD Biosciences) and anti-TNFα V450 (1:400 dilution, clone Mab11, BD Biosciences), then acquired on LSRII flow cytometer. Flow cytometry data was analyzed using FlowJo software. The HIV-1 $B57_{TW10}$ CD8$^+$ T cell clones, A16 and 457, were assessed for functionality toward cognate peptide by (i) staining of cell surface anti-CD3 V450 and anti-CD137 APC (BD Biosciences) for 20 min and then analyzed on the FACSCanto II (BD) according to standard procedures, and (ii) functional cytotoxicity against single HLA expressing K562 cell line loaded with cognate peptide, using target cell 7-AAD uptake as readout, as previously published (27). This study shows the data for A16, with 457 being published elsewhere (20). Gating strategies are shown in **Supplementary Figure 2**. Tetramers were produced in-house by refolding soluble HLA α-heavy chain-BirA and β2-microglobulin with peptide to create monomers, which were then conjugated at a 4:1 molar ratio to streptavidin-PE or -APC (Life Technologies) (24).

αβTCR Identification

Virus-specific CD8$^+$ T cells lines were incubated with 1 μM peptide or relevant peptide-pulsed C1R transfected cells for 2 h before detection of cytokine secretion using an anti-IFNγ antibody (IFNγ Secretion Assay Detection Kit APC; Miltenyi Biotec, Auburn, CA) as previously described (28). CD8$^+$ T cells were single-cell sorted directly into semi-skirted 96-well plates (Bio-Rad Laboratories Inc., USA) based on tetramer specificity and ± IFNγ production (FACSAria I, BD Biosciences operated by FlowCore, Monash University). Sorted plates were immediately stored at −80°C until required. TCR analysis of paired complementarity determining region (CDR)3α and β loops were carried out using multiplex nested RT-PCR and sequencing of α and β gene products as previously described (29). For virus-specific CD8$^+$ T cell clones, αβTCR usage was determined by DNA Sanger sequencing using either TCR-specific PCR for HD9G6 (30) or next-generation sequencing using published primer sequences (31) for A16 and 457.

TCR Expression in SKW3.hCD8αβ Cells

Full-length human TCRα and TCRβ cDNA was cloned into a self-cleaving 2A peptide-based pMIG vector as described previously (32). HEK293T packaging cells were incubated with 4 mg pEQ-pam3(-E) and 2 mg pVSV-G packaging vectors, in the presence of 4 mg pMIG vector each containing a specific TCR transgene using Lipofectamine 3000 (Life Technologies). HEK293T cell culture supernatant containing virus particles carrying the TCR transgene was then used to retrovirally transduce GFP-tagged SKW3.hCD8αβ cells or GFP-tagged SKW3.hCD8αβ.CD3 [for LTR5 TCR only (28)], which are negative for endogenous TCRαβ but contain CD3 and signaling components, as previously described (28). SKW3.hCD8αβ.TCR (hereafter referred to as SKW3) cell lines were maintained in RF10. Routine monitoring of TCR cell surface expression on SKW3 transduced cells was performed using anti-CD3 PE-Cy7 (1:500 dilution, clone SK7, BD Biosciences), anti-CD8 PerCP Cy5.5 (1:20 dilution, clone SK1, BD Biosciences) and GFP. Gating strategy shown in **Supplementary Figure 3**. Activation of SKW3.TCRs were assessed via cell surface staining with anti-CD3 PE-Cy7 (1:500 dilution, clone SK7, BD Biosciences), anti-CD8 PerCP Cy5.5 (1:20 dilution, clone SK1, BD Biosciences) and anti-CD69 APC (1:50 dilution, clone L78; BD Biosciences) following 16–20 h incubation with stimuli at 37°C, 5% CO_2. A representative gating strategy for SKW3.HC5 is shown in **Supplementary Figure 4**, with CD69 mean fluorescence intensity (MFI) values calculated after gating on FSC vs. SSC, single cells, GFP$^+$ cells, live cells, CD3$^+$CD8$^+$ cells and then CD69$^+$ cells.

Statistical Analysis

Statistical significance was determined by non-parametric one-way ANOVA (Kruskal-Wallis test) with *post-hoc* Dunn's multiple comparison test or unpaired Student's *t*-test using Prism 8 (GraphPad) with $*p < 0.05$, $**p < 0.01$, and $****p < 0.0001$. Error bars indicate the mean ± SEM.

RESULTS

Generation of Virus-Specific CD8$^+$ T Cell Lines and Clones

Virus-specific CD8$^+$ T cells can be generated following stimulation with viral cognate peptide-pulsed autologous PBMCs. To demonstrate both specificity and functionality, *in vitro* expanded T cell lines or clones were co-stained with anti-CD8 and tetramer phenotypic markers for identification of virus-specific T cells. As expected, variations in the frequency of expanded tetramer$^+$CD8$^+$ T cell lines were observed for both EBV and CMV (**Figures 1A,B**, middle panels), ranging between 32.6–90.7% ($n = 6$) and 6.15–74.0% ($n = 2$) of the total CD8$^+$ T cell population for $B7_{RPP}$ and $A2_{NLV}$, respectively. In addition, CD8$^+$ T cell clones raised against the HIV-1 $B57_{TW10}$ epitope in patients A16 and 457 showed

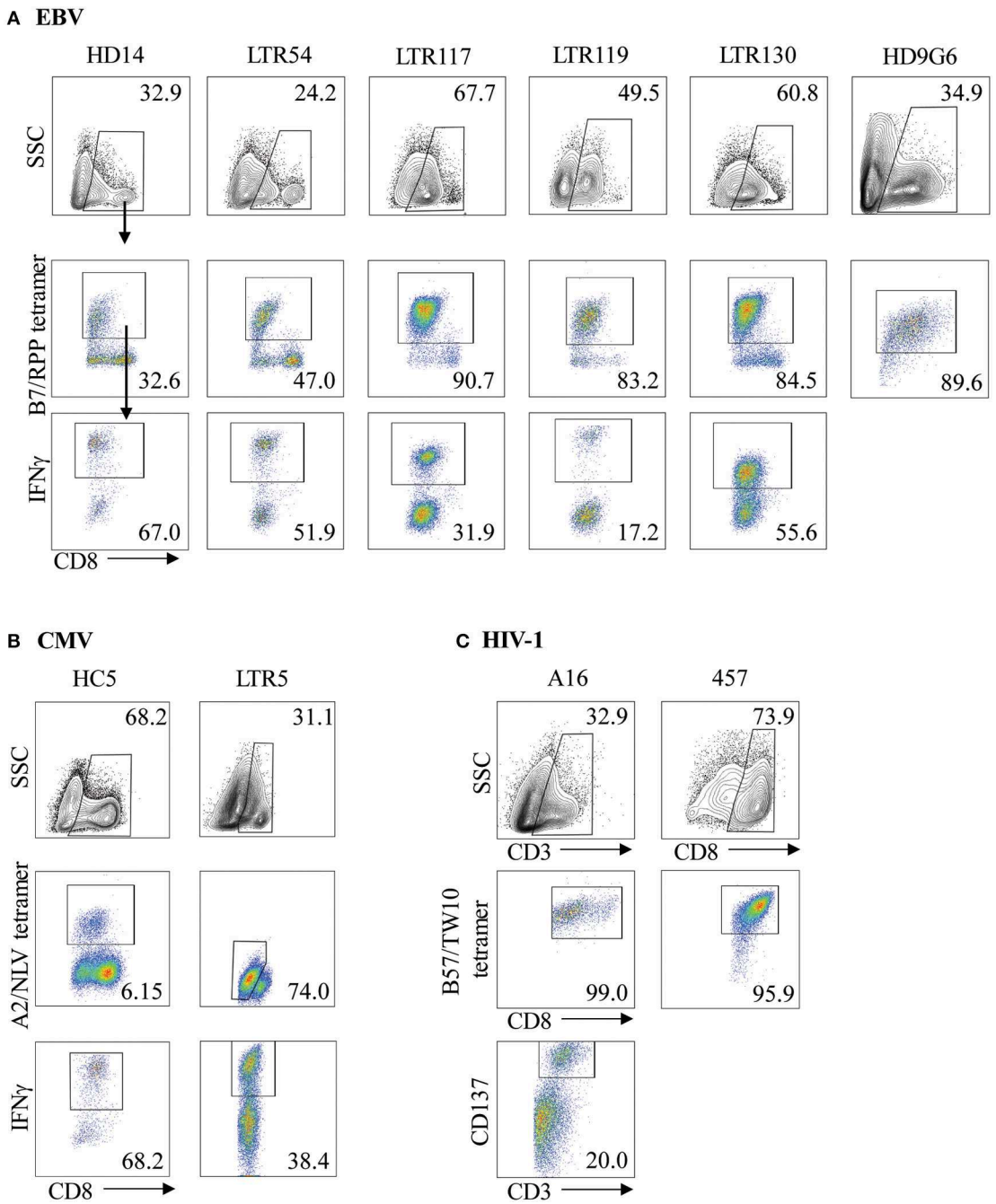

FIGURE 1 | Characterization of virus-specific CD8$^+$ T cells. Virus-specific CD8$^+$ T cell lines and clones for **(A)** EBV, **(B)** CMV, and **(C)** HIV-1 were examined for specificity following either re-stimulation with HLA-restricted APCs pulsed with cognate viral peptide or bulk PBMC sorting, using both anti-CD8 and specific tetramer. The functionality of virus-specific CD8$^+$ tetramer$^+$ T cells was assessed either using IFNγ production for T cell lines or via the CD137 activation marker for HIV-1 T cell clones. Cells were gated on FSC vs. SSC, single cells, CD8$^+$, CD8$^+$tetramer$^+$, CD8$^+$IFNγ$^+$ cells. Representative plots are shown.

very high frequencies following tetramer-specific PBMC bulk sorting (**Figure 1C**, middle panels), which were similar to the high frequencies observed against the EBV-B7$_{RPP}$ epitope (i.e., HD9G6). To assess the functionality of the EBV- or CMV-specific CD8$^+$ T cell lines to produce the pro-inflammatory cytokine IFNγ, cells were restimulated with HLA-restricted APCs pulsed with cognate viral peptide. The frequency of IFNγ production ranged from 17.2 to 67.0% and 38.4 to 68.2% of the CD8$^+$ tetramer$^+$ T cell population for B7$_{RPP}$ and A2$_{NLV}$, respectively (**Figures 1A,B**, lower panels). For HD9G6, the functionality of this B7$_{RPP}$-specific CD8$^+$ T cell clone is published elsewhere (21). For B57$_{TW10}$-specific CD8$^+$ T cell

clones A16 and 457, the activation marker CD137, which induces downstream effects of proliferation and cytolytic activity, was used to assess functionality when stimulated with cognate TW10 peptide (**Figure 1C**), with data for 457 reported elsewhere (20).

Increased Sensitivity for TCR Cross-Reactivity Detection Using SKW3 Reporter Cells

We have previously reported that CMV-specific $CD8^+$ T cells raised against $A2_{NLV}$ were differentially cross-reactive toward three HLA-B27 allotypes (B*27:07 > B*27:09 > B*27:05). These T cells were also shown to remain relatively stable following lung transplantation, but increased significantly in response to CMV reactivation (4, 11). Further characterization of the cross-reactive $A2_{NLV}$-specific TCR repertoires from two unrelated individuals showed a striking similarity for the cross-reactive TCR clonotype. Additionally, this study also demonstrated that expression of cross-reactive TCRs in SKW3 cells was a robust system that maintains specificity without the need for continuous *in vitro* expansion of T cell lines or clones for further functional immunoassays (28). In this study, we extended the HLA-B27 allotype panel (B*27:01–B*27:10) to map the immunogenic

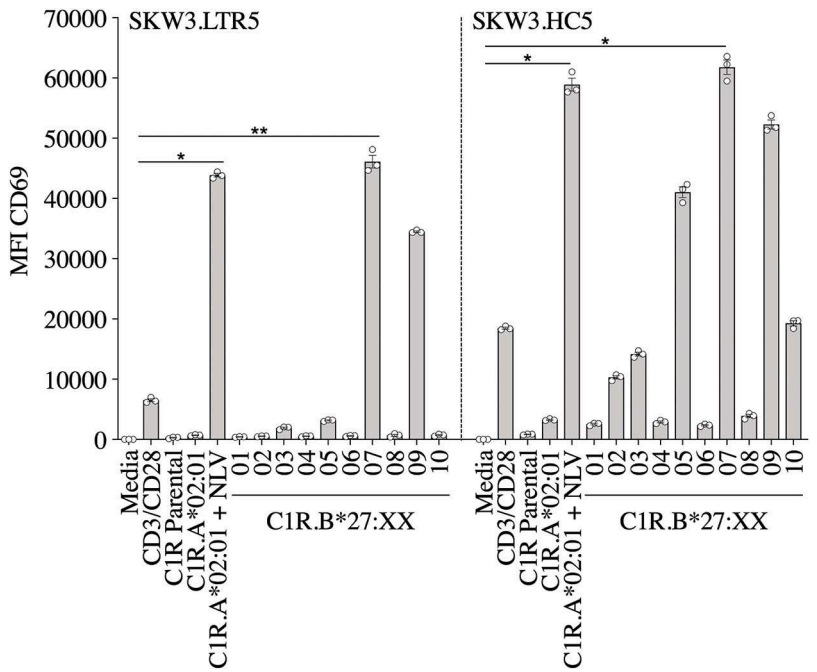

FIGURE 2 | Activation of SKW3.$A2_{NLV}$ TCR cells by HLA-B27–expressing APCs. SKW3.TCR activation was measured using cell surface CD69 upregulation after 16–20 h stimulation with C1R.A*02:01 ± cognate NLV peptide and a panel of C1R.B27 transfectants. CD69 MFI values were calculated after gating on FSC vs. SSC, single cells, GFP^+ cells, live cells, $CD3^+CD8^+$ cells then $CD69^+$ cells. Mean ± SEM are shown (a single experiment with triplicate data is shown from independent biological replicates performed at least twice). Statistical significance denoted by $^*p < 0.05$ and $^{**}p < 0.01$ was determined by repeated measures non-parametric ANOVA (Kruskal–Wallis test) with *post-hoc* Dunn's multiple comparison test.

TABLE 1 | Virus-specific αβTCR signatures.

Virus and HLA/epitope	TCR	α-chain			β-chain			References
		TRAV	CDR3	TRAJ	TRBV	CDR3	TRBJ	
EBV B7 $_{RPP}$	LTR54.1	38-1	CAFSYNNNDMRF	43	4-1	CASSQETGIYTQYF	2-3	
	LTR54.2	38-1	CAFIQGAQKLVF	54	4-1	CASSQEAFNYEQYF	2-7	
	LTR117	38-1	CAFASSNTGKLIF	43	4-1	CASSQDIWTSGYTF	2-3	
	LTR119	38-2/DV8	CALGGGAQKLVF	54	28	CASRLGLGDREDEKLFF	1-4	
	HD9G6	14/DV4	CAMRDDTGGFKTIF	9	19	CASSISSGVAYEQYF	2-7	(21)
CMV A2 $_{NLV}$	HC5	3	CAVRGTNARLMF	31	12	CASSSVNEAFF	1-1	(28)
	LTR5	3	CAVRNNNARLMF	31	12	CASSIVNEAFF	1-1	(28)
HIV-1 B57 $_{TW10}$	A16.1	4	CLVGEVRGGFKTIF	9	4-3	CASSQARGGAETQYF	2-5	
	A16.2	4	CLVGGEDYKLSF	20	4-3	CASSQARGGAETQYF	2-5	
	457	39	CAVDINTSGTYKYIF	40	10-3	CAISRQGARQETQYF	2-5	(20)

profiles of cross-reactive virus-specific T cells (SKW3.LTR5 and SKW3.HC5), which were previously derived from two HLA-A2$^+$ donors recognizing both the CMV A2$_{NLV}$ epitope and HLA-B27 molecules (28). Stimulation of both SKW3.LTR5 and SKW3.HC5 with our new panel of C1R.B27 allotypes reconfirmed our previous findings of an immunogenic hierarchy (B*27:07 > B*27:09 > B*27:05) but also revealed additional cross-reactivity toward B*27:03 for SKW3.LTR5 and B*27:10 > B*27:03 > B*27:02 for SKW3.HC5. All negative, background (media and C1R Parental, C1R.A*02:01) and positive (CD3/CD28 beads, C1R.A*02:01+NLV) controls were as expected (**Figure 2**).

Given the utility of SKW3 reporter cells for profiling TCR cross-reactivity, we adopted this approach to further explore HLA-B27 allorecognition patterns by immunodominant HLA-restricted virus-specific T cells. Here, several cognate peptide-specific CD8$^+$ T cells identified using either the ICS immunoassay (i.e., T cell lines) or tetramer sorting (i.e., T cell clones) were sequenced for paired TCR α and β chains. The highest frequency αβTCR was then selected for retrovirus transduction into SKW3 cells (**Table 1**). Following transduction, extremely high levels of clonality of >90% were easily achieved and maintained by sorting the top 10% of GFP$^+$CD3$^+$ cells if TCR expression decreased during long-term sub-culturing (**Figure 3**).

Dissection of Virus-Specific TCR Cross-Reactivity Toward HLA-B27 Allotypes Reveals Distinct Patterns of Allorecognition

Here we investigated whether EBV-B7$_{RPP}$-specific CD8$^+$ T cells could cross-recognize HLA-B27 molecules as a potential trigger of allorecognition. Following validation with the cognate peptide, we re-stimulated our day 13 *in vitro*-expanded B7$_{RPP}$-specific CD8$^+$ T cells, which were generated from five EBV-seropositive individuals (HD14, LTR54, LTR117, LTR119, and LTR130), against a panel of HLA-B27-expressing APCs in a 6 h ICS assay with functionality assessed via Th1 cytokine production (i.e., TNFα$^+$ or IFNγ$^+$ alone or dual TNFα$^+$IFNγ$^+$). Specificity of the B7$_{RPP}$-specific CD8$^+$ T cells was confirmed in all individuals by reactivity to C1R.B*07:02 in the presence of cognate RPP peptide. Remarkably, differential patterns of HLA-B27 allorecognition were observed across these individuals. Here, B7$_{RPP}$-specific CD8$^+$ T cells from HD14 demonstrated a moderate response to B*27:02 and weak responses to B*27:08, LTR54 showed a very dominant response to B*27:08 only, LTR117 recognized several allotypes with B*27:02 > B*27:01 > B*27:07 and similar levels for B*27:03/08/09, LTR119 weakly recognized B*27:02/08 and LTR130 moderately responded to B*27:08. All negative, background (media and C1R Parental, C1R.B*07:02) and positive (CD3/CD28 beads) controls were as expected (**Figure 4A**, **Table 2**). Therefore, for the first time, we report a new model of EBV/HLA-B27 cross-reactivity that was observed across multiple individuals.

To explore these HLA-B27 allorecognition patterns in greater depth we generated SKW3.TCR cells expressing the B7$_{RPP}$-specific TCR observed at the highest frequency for LTR54 (LTR54.1 and LTR54.2), LTR117 and LTR119, in addition to the published HD9G6 TCR, which showed cross-reactivity toward HLA-B*40:01 (21) (**Table 1**). For LTR54, two TCRs with the same α and β-chain variable regions but different junction regions and CDR3 loops were observed. To investigate whether these contrasting regions were pivotal for allorecognition both TCRs were expressed in SKW3 cells. As observed with the immunogenic hierarchies of CMV A2$_{NLV}$ cross-reactive TCRs for HC5 and LTR5, we demonstrated greater sensitivity of allorecognition using SKW3.TCR reporter cells. The patterns were as follows: LTR54.1 strongly recognized B*27:08 and to a weaker extent B*27:02/07/04/06/09, whilst in comparison LTR54.2 also strongly responded to B*27:08 but weakly recognized alternate B27 allotypes B*27:06/01/02/04/07; LTR117 recognized most of the allotypes with the strongest

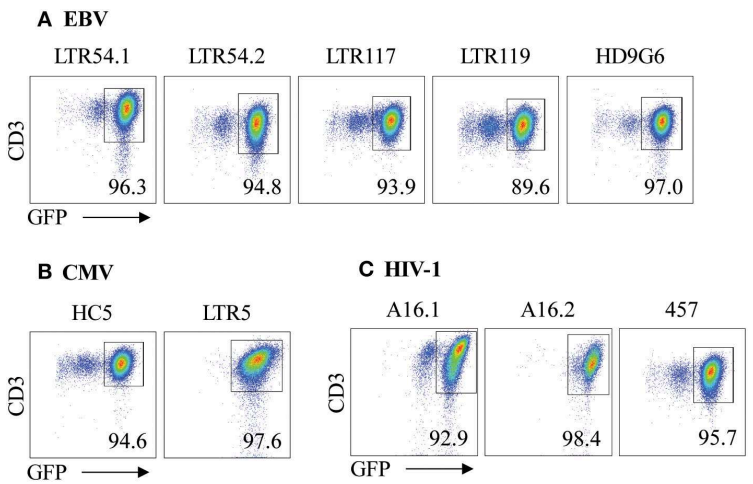

FIGURE 3 | αβTCR expression of SKW3 reporter cells. Retrovirally transduced SKW3 cells expressing cross-reactive virus-specific αβTCRs for **(A)** EBV, **(B)** CMV, and **(C)** HIV-1 were monitored for stable cell surface TCR expression. Cells were gated on FSC vs. SSC, single cells, GFP$^+$CD3$^+$ cells. Representative plots are shown.

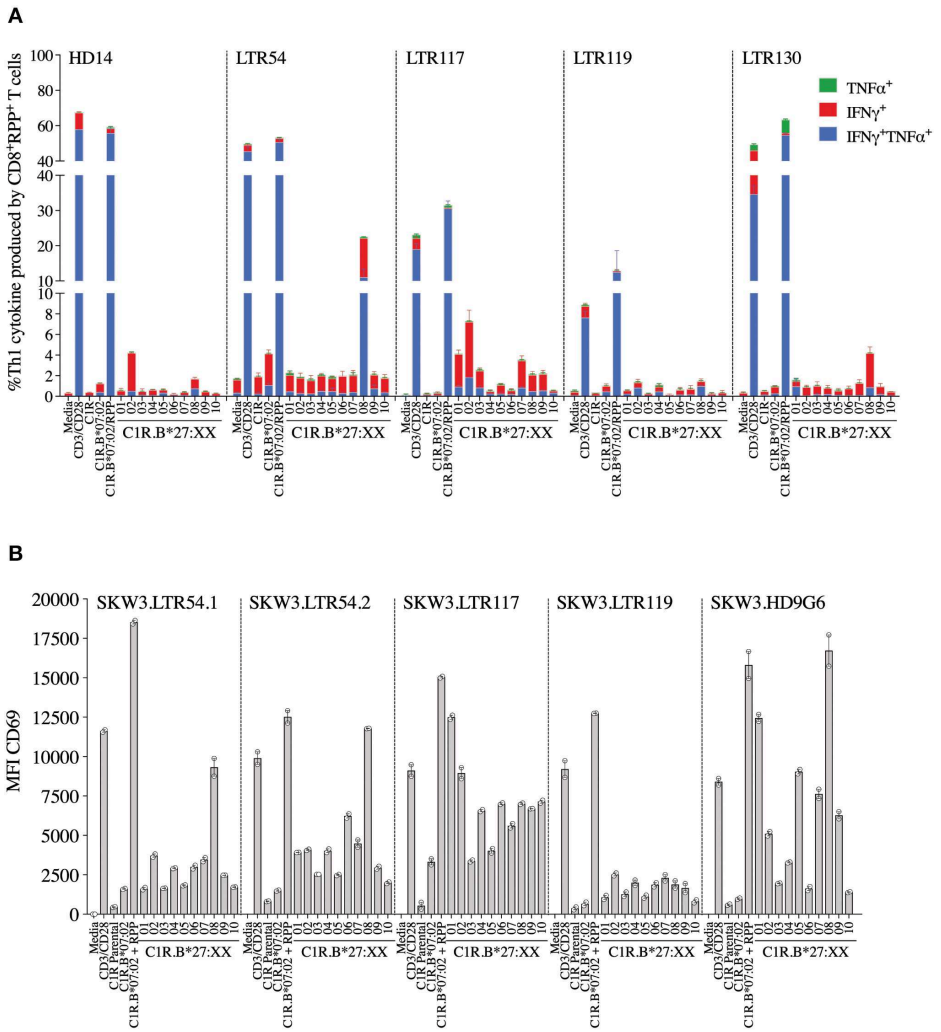

FIGURE 4 | EBV $B7_{RPP}$ allorecognition of HLA-B27 molecules. **(A)** Day 13 *in vitro* expanded $B7_{RPP}$-specific $CD8^+$ T cells were stimulated with C1R.B*07:02 ± cognate RPP peptide and a panel of C1R.B27 transfectants before performing a 6 h ICS, with T cell responses measured by the production of Th1 cytokines (i.e., $TNF\alpha^+$ or $IFN\gamma^+$ alone or dual $TNF\alpha^+IFN\gamma^+$) after gating on $CD8^+$tetramer$^+$ T cells. **(B)** SKW3.TCR activation was measured using cell surface CD69 upregulation after 16–20 h stimulation with C1R.B*07:02 ± cognate RPP peptide and a panel of C1R.B27 transfectants. CD69 MFI values were calculated after gating on FSC vs. SSC, single cells, GFP^+ cells, live cells, $CD3^+CD8^+$ cells then $CD69^+$ cells. Mean ± SEM are shown (single experiments with duplicate data for ICS assay and CD69 assay are shown from independent biological replicates each performed at least twice).

toward B*27:01/02 followed by B*27:10/08/04/09/06/07; LTR119 showed weak responses across several allotypes with B*27:02/07 followed by B*27:08/04; and finally HD9G6 strongly recognized B*27:08/01 then B*27:05/07/09/02/04/03 (**Figure 4B**, **Table 2**).

A recent report highlighted that HIV-1-specific memory T cells generated from Gag $B57_{TW10}$ epitope can mediate abacavir-induced hypersensitivity reactions through molecular mimicry (20). Therefore, we explored the alloreactive potential of $B57_{TW10}$-specific $CD8^+$ T cells toward HLA-B27. TCRs from two T cell clones (A16 and 457) raised against the Gag $B57_{TW10}$ epitope were expressed in SKW3 cells for functional evaluation. Sequencing of the A16 T cell clone revealed two α-chains with different junction regions and CDR3 loops, therefore both TCRs were independently expressed in SKW3 cells (**Table 1**). Strikingly, comparisons of SKW3.A16.1 and SKW3.A16.2 show a 9-fold difference in recognition of C1R.B*57:01 cells presenting cognate TW10 peptide (positive control), suggesting that A16.2 TCR is the primary driver of the cognate peptide recognition. Yet despite this, SKW3.A16.1 recognizes both B*27:07 and B*27:05 at a similar magnitude to B*27:05 allorecognition by SKW3.A16.2. For SKW3.457, responses were biased toward B*27:01 and B*27:02 (**Figure 5**, **Table 2**). Furthermore, we examined whether immunodominant IAV $A2_{GIL}$-specific $CD8^+$ T cells, from an alternate RNA virus that induces acute viral infection, could also alloreact toward HLA-B27 allotypes. Here, a total of six healthy donors were screened, and interestingly no significant allorecognition was observed above background levels (**Supplementary Figure 5**).

TABLE 2 | Comparison of T cell cross-reactivity using cellular immunoassays.

Virus and HLA/Epitope	TCR	Assay	B*27:01	B*27:02	B*27:03	B*27:04	B*27:05	B*27:06	B*27:07	B*27:08	B*27:09	B*27:10
EBV $B7_{RPP}$	HD14	T cells	0.57	3.62	0.95	0.86	0.51	0.46	1.19	1.53	0.38	0.41
	LTR54	T cells	1.23	1.12	0.81	1.28	0.97	1.11	1.18	18.55	1.39	0.92
	LTR54.1	TCR	1758.00	3854.50	1784.50	3052.50	1963.00	3125.00	3594.50	9457.00	2614.00	1854.00
	LTR54.2	TCR	4415.00	4576.00	3013.50	4519.50	2974.00	6729.50	4975.00	12277.50	3436.50	2489.00
	LTR117	T cells	3.27	6.12	1.93	0.33	0.73	0.38	2.71	1.44	1.55	0.41
		TCR	12922.00	9376.00	3804.00	7001.50	4451.00	7444.00	6037.00	7436.50	7119.50	7568.00
	LTR119	T cells	0.39	0.99	0.06	0.72	0.09	0.28	0.41	1.18	0.12	0.15
		TCR	1781.00	3240.00	1985.50	2713.50	1833.50	2585.00	3024.50	2602.00	2367.50	1534.50
	LTR130	T cells	1.39	0.80	0.91	0.71	0.46	0.68	1.12	3.99	0.85	0.37
	HD9G6	TCR	13144.00	5796.00	2654.00	3980.00	9737.00	2321.00	8331.00	17422.50	6972.00	2093.00
CMV $A2_{NLV}$	HC5	T cells	0.76	4.70			2.42		15.70		7.11	
		TCR	3815.67	11587.33	15446.33	4199.33	42312.67	3642.00	63057.00	5168.33	53564.00	20551.33
	LTR5	T cells		0.30	0.40	0.40	3.80		26.40		22.20	
		TCR	1197.33	1338.33	2717.33	1345.00	3973.00	1366.33	46910.67	1505.67	35309.33	1546.00
HIV-1 $B57_{TW10}$	A16.1	TCR	2644.00	2947.00	3620.50	2362.00	7335.00	2596.00	7858.00	2592.50	3683.00	1820.50
	A16.2	TCR	2886.50	3139.00	4769.00	2113.00	6734.00	2173.00	4110.50	2988.50	3838.50	2385.50
	457	TCR	6959.50	13991.00	1638.00	2030.50	2003.50	2094.00	2641.50	2533.00	2175.00	1612.00

Assays: ICS for T cells, CD69 upregulation for TCR.

Cognate Viral Peptide Presented by HLA-B27 Allotypes Does Not Confer Additional Immunogenicity

To demonstrate that virus-specific TCR cross-reactivity toward HLA-B27 allotypes is *bona fide* and cannot be influenced by the presence of cognate peptide we examined the immune response of SKW.HC5 (i.e., CMV $A2_{NLV}$), SKW3.LTR54.1 (i.e., EBV $B7_{RPP}$), and SKW3.457 (i.e., HIV-1 $B57_{TW10}$) toward a panel of HLA-B27 stimulators in the absence and presence of cognate peptide. All SKW3.TCR lines generated responses to negative, background (media and C1R Parental, C1R.A*02:01 or C1R.B*07:02 or C1R.B*57:01) and positive (CD3/CD28 beads and C1R.A*02:01+NLV or C1R.B*07:02+RPP or C1R.B*57:01+TW10) controls as expected. Importantly, no differences in the magnitude of HLA-B27 allorecognition was observed between APCs in the presence or absence of cognate peptide across all three SKW3.TCR lines. However, surprisingly a statistically significant difference was determined following stimulation of SKW.HC5 with C1R.B*27:05+NLV ($p < 0.0001$), which could indicate a role for the presented peptide, however this requires further confirmation (**Figure 6**).

DISCUSSION

In this study, we examined the cross-reactive potential of $CD8^+$ T cells specific for immunodominant epitopes derived from

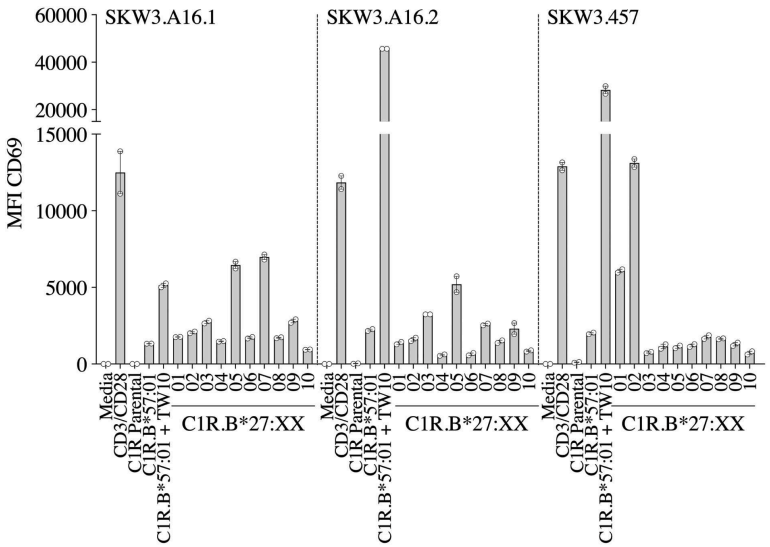

FIGURE 5 | HIV-1 $B57_{TW10}$ allorecognition of HLA-B27 molecules. TCR activation was measured using cell surface CD69 upregulation after 16–20 h stimulation with C1R.B*57:01 ± cognate TW10 peptide and a panel of C1R.B27 transfectants. CD69 MFI values were calculated after gating on FSC vs. SSC, single cells, GFP^+ cells, live cells, $CD3^+CD8^+$ cells then $CD69^+$ cells. Mean ± SEM are shown (a single experiment with duplicate data is shown from independent biological replicates performed at least twice).

three different chronic viruses (i.e., CMV, EBV, and HIV-1), presented by commonly expressed HLA (i.e., A2, B7, and B57). We demonstrated that these virus-specific $CD8^+$ TCRs were capable of vigorous cross-reactivity toward specific HLA-B27 allotypes, and that the immune responses were hierarchical and varied considerably across the three chronic viruses.

Whilst, we previously reported a defined pattern of strong HLA-B27 T cell cross-reactivity (B*27:07 > 09 > 05) by CMV $A2_{NLV}$ $CD8^+$ TCRs for both LTR5 and HC5 (11, 28), this study extended the number of B27 subtypes examined and revealed additional cross-reactivity toward B*27:10 > 03 > 02 for HC5. Interestingly, despite subtle sequence differences in the CDR3 regions of both the α- and β-chains (2 and 1 amino acids, respectively) between LTR5 and HC5, the fine specificity of strong TCR interactions with B*27:07/09 allotypes were maintained. The data suggests that the composition of the allopeptide(s) presented by each HLA-B27 allomorph are similar or alternatively, of high affinity and that molecular flexibility of the CDR3 loops aids promotion of TCR engagement (33, 34). In contrast, weaker responses toward B*27:02/03/10 show delineation in TCR interaction, with LTR5 not demonstrating recognition of these allotypes, which may be due to weak TCR interactions below the assay sensitivity threshold. This suggests that the allopeptide contribution required to form the ternary complex is impacted by the variability observed in the CDR3 regions, which is supported by structural studies of the murine 2C TCR demonstrating that variations in the CDR3α loop dictated TCR affinity and cross-reactivity between distinct ligands (35). Indeed, the importance of the TCR variable domains in promoting high affinity interactions with pHLA complexes was also shown with the human HLA-A2-restricted cancer antigen MART-1 (36). Further investigations are required to decipher the allopeptide(s) presented by these HLA-B27 allotypes and determine their exact role in conferring cross-reactivity.

We next examined the magnitude of cross-reactivity exhibited by EBV-specific $B7_{RPP}$ $CD8^+$ T cells toward HLA-B27 allotypes. In the five $HLA\text{-}B7^+$ individuals, including a healthy donor and immunosuppressed patients, allorecognition resulted in production of proinflammatory Th1 cytokines (IFNγ and TNFα) mainly toward either B*27:02 or B*27:08. Although, it should be noted that an additional screen of four healthy donors showed no HLA-B27 cross-reactivity, suggesting that allorecognition is driven by private TCR usage. The $B7_{RPP}$ $CD8^+$ TCR repertoires were sequenced for three of these individuals to determine their clonotypic profiles. Interestingly, only two clonotypes were observed for LTR54 (i.e., LTR54.1 and LTR54.2), which differed in the CDR3 and J regions of both TCRα- and β-chains. Both TCRs were expressed in SKW3 cells for further functional validation. Additionally, comparison of the $B7_{RPP}$ $CD8^+$ TCR clonotypes showed a high degree of similarity between LTR54.1 and LTR117, with differences only noted in the CDR3α- and β-loops. Whilst, LTR119 and the previously reported $B7_{RPP}$ $CD8^+$ T cell clone, HD9G6 (21), are vastly different from the other TCRs in this cohort. Interestingly, the strongest TCR cross-reactivity was relatively restricted to B*27:08 (LTR54.1, LTR54.2, HD9G6) and B*27:02 (LTR117), although there was a degree of allorecognition toward other subtypes for most TCRs. These observations highlight that both private (i.e., LTR119 and HD9G6) and shared (i.e., LTR54.1 and LTR117) TCR specificities contribute to cross-reactivity, and that the cross-reactive pattern diversity is dependent on the Vβ region (2, 15, 16). Furthermore, Amir et al. (2) also reported that T cell clones with identical Vβ regions from the same individual held private specificities and generated different alloreactions. For example, in donor BDV

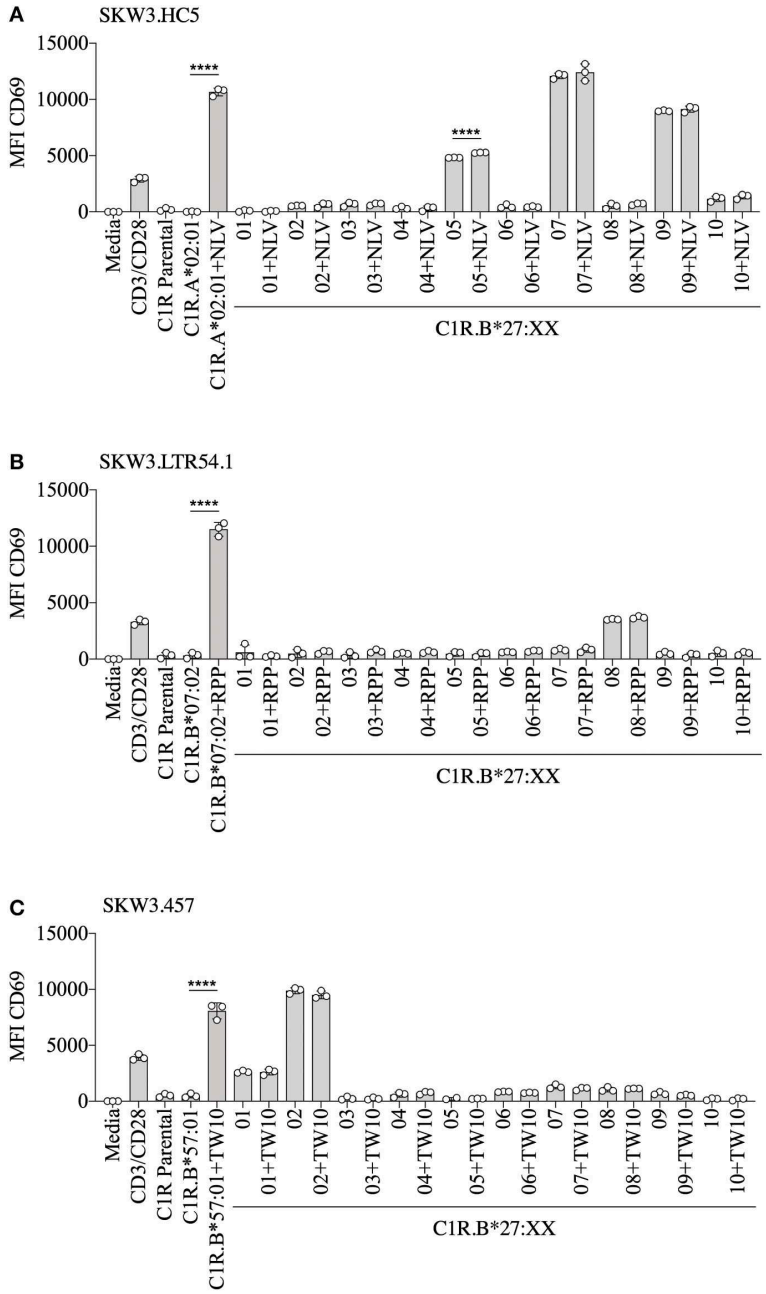

FIGURE 6 | HLA-B27 presentation of cognate viral peptide does not confer additional immunogenicity. **(A)** SKW.HC5 (i.e., CMV $A2_{NLV}$), **(B)** SKW3.LTR54.1 (i.e., EBV $B7_{RPP}$), and **(C)** SKW3.457 (i.e., HIV-1 $B57_{TW10}$) TCR activation was measured using cell surface CD69 upregulation after 16–20 h stimulation with HLA-restricted C1R transfectants ± cognate peptide and a panel of C1R.B27 transfectants. CD69 MFI values were calculated after gating on FSC vs. SSC, single cells, GFP^+ cells, live cells, $CD3^+CD8^+$ cells then $CD69^+$ cells. Mean ± SEM are shown (single experiment with triplicate data). Statistical significance using unpaired Student's *t*-test is denoted by $^{****}p < 0.0001$.

a T cell clone raised against CMV $B7_{RPHERNGFTVL}$ with TCR Vβ7.2 recognized DRB1*08:01, whilst another T cell clone from same individual with the identical Vβ did not. Additionally, in donor FKR an influenza $A2_{GIL}$ T cell clone with Vβ17 recognized allogeneic HLA-B*64:01 but another T cell clone with the identical Vβ failed. These T cell clones had private differences in TCR sequence, which effectively abrogated alloreactivity.

For the herpesvirus TCRs, the allorecognition hierarchy remained relatively static for the strongest responses, but this was not observed in the case of HIV-1 $B57_{TW10}$ $CD8^+$ TCRs in that 457 and A16 TCRs were completely focused toward different HLA-B27 allotypes. Here, we show that 457 TCR cross-reacted strongly toward B*27:02 > 01, with two TCRs derived from A16 strongly recognizing B*27:05/07 for A16.1 and B*27:05 for A16.2.

Comparison of their TCRs revealed that their signatures were completely different, supporting that the $B57_{TW10}$ specificity is driven by private TCR usage in these two individuals. Particularly of interest was the dual expression of two different TCRα-chains from the A16 T cell clone, which when independently expressed in SKW3 reporter cells, showed reactivity differences not only toward the B27 subtypes but also importantly against the cognate antigen. We observed that the A16.2 TCR was geared toward cognate antigen recognition, with the A16.1 TCR being more alloreactive. Up to 30% of human peripheral T cells naturally express dual TCRα-chains (37), with multiple studies demonstrating that the allelic inclusion facilitates a heightened immune response by providing an additional chance for antigen recognition and engagement [extensively reviewed in (38)].

So, what drives the preferential HLA-B27 allorecognition displayed by these virus-specific TCRs? Undoubtedly, the polymorphic nature of the B27 molecule itself greatly influences the peptide cargo being displayed to surveying T cells (**Figure 7**). In our study, the $A2_{NLV}$ $CD8^+$ TCRs preferentially bind to B*27:07/09/05, which differ by 1 (B*27:09) and 5 (B*27:07) amino acids compared to the consensus B*27:05 allotype. These polymorphisms directly impact the D/E (position 114, peptide contacts P5–P7) and F (position 116, peptide contact P9) peptide-binding pockets, which are known immunological hot spots for non-permissive HLA mismatches in transplantation (39–43). Given that the public $A2_{NLV}$ $CD8^+$ TCR co-recognizes these three molecules and their relative impact on the peptide-binding pockets D/E and F, suggests that each may be presenting an alternate allopeptide with affinity above a threshold to promote TCR engagement. This is supported by the prototypic HLA-B8-restricted LC13 TCR which is capable of engaging with HLA-B*44:05 presenting either an allotype or mimotope (18). In addition, we cannot exclude that the same allopeptide may also be presented by all HLA-B27 molecules, with allorecognition being impacted by differences in conformational flexibility. Indeed, a study by Loll et al. demonstrated that a HLA-B27-derived self-peptide derived from vasoactive intestinal peptide receptor type 1 (epitope; RRKWRRWHL) is differentially presented by AS-associated B*27:04 and B*27:05 compared to the non-AS-associated B*27:06 and B*27:09 due to structural variations in molecular dynamics (44). For $B7_{RPP}$ $CD8^+$ TCRs, recognition was focused toward B*27:08/02/01, with B*27:01 and B*27:02 differing by a single amino acid (position 80) and both differing from B*27:08 by 5 amino acids (positions 77, 80–83), all of which also influence the F peptide-binding pocket (**Figure 7**). Finally, for the $B57_{TW10}$ $CD8^+$ TCRs we observed completely divergent recognition of B27 allotypes by 457 (B*27:01/02) and A16 (B*27:05/07). However, a common feature is involvement of the F pocket at positions 80 and 116, respectively. Interestingly, the F pocket not only determines the carboxy terminal motif of HLA-I peptides (45), but in other HLA-B27 allotypes has also been shown to affect anchoring sites (i.e., B*27:06; P3, PΩ-2, and PΩ) (46). Moreover, positions 114 and 116 are important for the chaperone tapasin, involved in loading of optimal peptides on HLA-I molecules (47, 48). Whilst, the identification of allopeptides has been a major limiting factor hampering translational impact in clinical studies, further investigations are warranted to assess the true impact of T cell cross-reactivity.

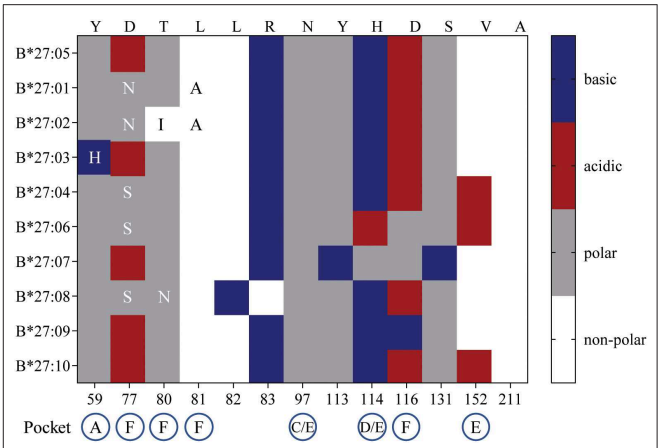

FIGURE 7 | HLA-B27 polymorphisms and amino acid chemical properties. B27:01-B*27:04, B*27:06-B*27:10 amino acid polymorphisms were compared to the consensus sequence for B*27:05. The chemical properties of substituted residues and their influence on HLA-I peptide-binding pockets are detailed.

Here we analyzed three HLA-B27 cross-reactivity models, including our newly identified EBV/HLA-B27 model, using T cell lines/clones and the more fine-tuned TCR-specific SKW3 cell lines, to reveal the diversity and breadth of cross-reactivity against different HLA-B27 allotypes. Specifically, we showed that cross-reactive TCRs (LTR5, HC5, LTR119, A16.1) derived from the three heterologous viruses were capable of recognizing B*27:07, with cross-reactive TCRs from two viruses recognizing either B*27:01 (LTR117, HD9G6, 457), B*27:02 (LTR119, 457), or B*27:05 (HC5, A16.1, A16.2). Collectively, this study demonstrated selective TCR cross-reactivity toward HLA-B27 allotypes by chronic latent viruses, which may evoke clinically relevant alloreactivity following transplantation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All study participants provided written consent, with ethics approval granted by The Alfred Hospital [Victoria, Australia; ethics no. 175/02, lung transplant recipient (LTR)5, LTR54, LTR117, LTR119, LTR130], Monash University (Victoria, Australia; ethics no. 10950, healthy control/donor HC5, HD14), Australian Bone Marrow Donor Registry (NSW, Australia; ethics no. 2012/05, healthy donors NM003, NM008, NM009,NM010, NM014, NM016), Royal Perth Hospital (Western Australia, Australia; ethics no. HREC 1999-021, HIV-1^+ patients A16, 457) and Leiden University Medical Center (Leiden, The Netherlands; buffy coat donation, HD9G6) in accordance

with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LR, HH, LD'O, and NM contributed conception and designed of the study. LR, HH, JS, LD'O, TN, and NM performed the experiments. LR, HH, LD'O, TN, and NM analyzed the data. FC, JR, TK, AP, and NM made significant contributions to reagents. LR and NM wrote the manuscript. All authors read 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.2020.00248/full#supplementary-material

Supplementary Figure 1 | HLA cell surface expression of APCs. APCs were stained with primary antibody either **(A)** pan-HLA-I W6/32 or **(B)** anti-HLA-B7/27 ME1, followed by secondary goat anti-mouse IgG PE. A secondary (2°) antibody alone control was used for background staining. MFI was calculated after gating FSC vs. SSC then primary antibody histogram. Representative plots are shown.

Supplementary Figure 2 | Gating strategy for specificity and functionality of virus-specific $CD8^+$ T cells. Representative virus-specific $CD8^+$ $tetramer^+$ T cells was assessed for either **(A)** IFNγ production with cells gated on FSC vs. SSC, single cells, live cells, $CD8^+$, $CD8^+tetramer^+$, and $CD8^+IFN\gamma^+$ cells or **(B)** CD137 activation with cells gated on FSC vs. SSC, $CD3^+$ or $CD8^+$, $CD8^+tetramer^+$, and $CD3^+CD137^+$ cells.

Supplementary Figure 3 | Gating strategy for SKW3.TCR expression. Representative cell surface TCR expression for SKW3.LTR119 is shown. Cells were gated on FSC vs. SSC, single cells, GFP^+CD3^+ cells.

Supplementary Figure 4 | Gating strategy for CD69 upregulation assay. Representative CD69 cell surface upregulation for SKW3.HC5 is shown following stimulation with media, C1R.A*02:01+NLV (cognate peptide), C1R.B*27:01 (non-cross-reactive B27 allele) and C1R. B*27:07 (cross-reactive B27 allele). CD69 MFI values were calculated after gating on FSC vs. SSC, single cells, GFP^+ cells, live cells, $CD3^+CD8^+$ cells, and then $CD69^+$ cells.

Supplementary Figure 5 | IAV $A2_{GIL}$ allorecognition of HLA-B27 molecules. **(A)** Representative gating strategy of NM003 d13 $A2_{GIL}$-specific $CD8^+$ T cells stimulated with C1R.A*02:01+GIL peptide; FSC vs. SSC, single cells, live cells, $CD8^+$, $CD8^+tetramer^+$ and $IFN\gamma^+TNF\alpha^+$ cells. **(B)** Day 13 *in vitro* expanded $A2_{GIL}$-specific $CD8^+$ T cells were stimulated with C1R.A*02:01 ± cognate GIL peptide and a panel of C1R.B27 transfectants before performing a 6 h ICS, with T cell responses measured by the production of Th1 cytokines (i.e., $TNF\alpha^+$ or $IFN\gamma^+$ alone or dual $TNF\alpha^+IFN\gamma^+$) after gating on $CD8^+tetramer^+$ T cells. Mean ± SEM are shown (single experiment with duplicate data).

Supplementary Table 1 | HLA class I typing of study participants.

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REVIEW
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Cross-Reactive Immunity Among Flaviviruses

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Flaviviruses consist of significant human pathogens responsible for hundreds of millions of infections each year. Their antigenic relationships generate immune responses that are cross-reactive to multiple flaviviruses and their widespread and overlapping geographical distributions, coupled with increases in vaccination coverage, increase the likelihood of exposure to multiple flaviviruses. Depending on the antigenic properties of the viruses to which a person is exposed, flavivirus cross-reactivity can be beneficial or could promote immune pathologies. In this review we describe our knowledge of the functional immune outcomes that arise from varied flaviviral immune statuses. The cross-reactive antibody and T cell immune responses that are protective versus pathological are also addressed.

Keywords: flavivirus, dengue, Zika, yellow fever, tick-borne encephalitis, cross-protection, vector-borne

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INTRODUCTION TO FLAVIVIRUSES

Flaviviruses are enveloped single-stranded positive-sense RNA viruses that share conserved structural and genomic features (1). The viral genome encodes for three structural and seven non-structural proteins that are needed for virus replication and assembly (1). Some of the most prominent mosquito-borne flaviviral human pathogens include the hemorrhagic fever viruses, dengue (DENV) and yellow fever (YFV), and neurotropic viruses, such as West Nile (WNV), Japanese encephalitis (JEV), Saint Louis encephalitis (SLEV), and Zika (ZIKV). Yet other flaviviruses that are human pathogens, such as Kyasanur forest disease (hemorrhagic) and Powassan (encephalitic) viruses, are tick-borne. Additional flaviviruses have no known vector (2) while others are thought to be restricted to insects or bats and are not reported to cause human disease (3). Phylogenetic analysis has shown that flaviviruses cluster in genomic similarity according to their dominant vector (**Figure 1**), which also is a major contributing factor to the often-overlapping global distribution patterns of each flavivirus. The genetic differences amongst flaviviruses result in both conserved and species-specific attributes, such as cellular and tissue tropism upon infection and, importantly for the purposes of this review, antigenic properties.

CLASSIFICATION AND ANTIGENIC RELATIONSHIPS AMONG FLAVIVIRUSES

The name flavivirus (flavus- means "yellow" in Latin) stems from early research done on the YFV vaccine in 1930s, for which a Nobel Prize was awarded to Marx Theiler in 1951 (4). In the initial classification scheme, arthropod-borne viruses were classified based on their ability to replicate and

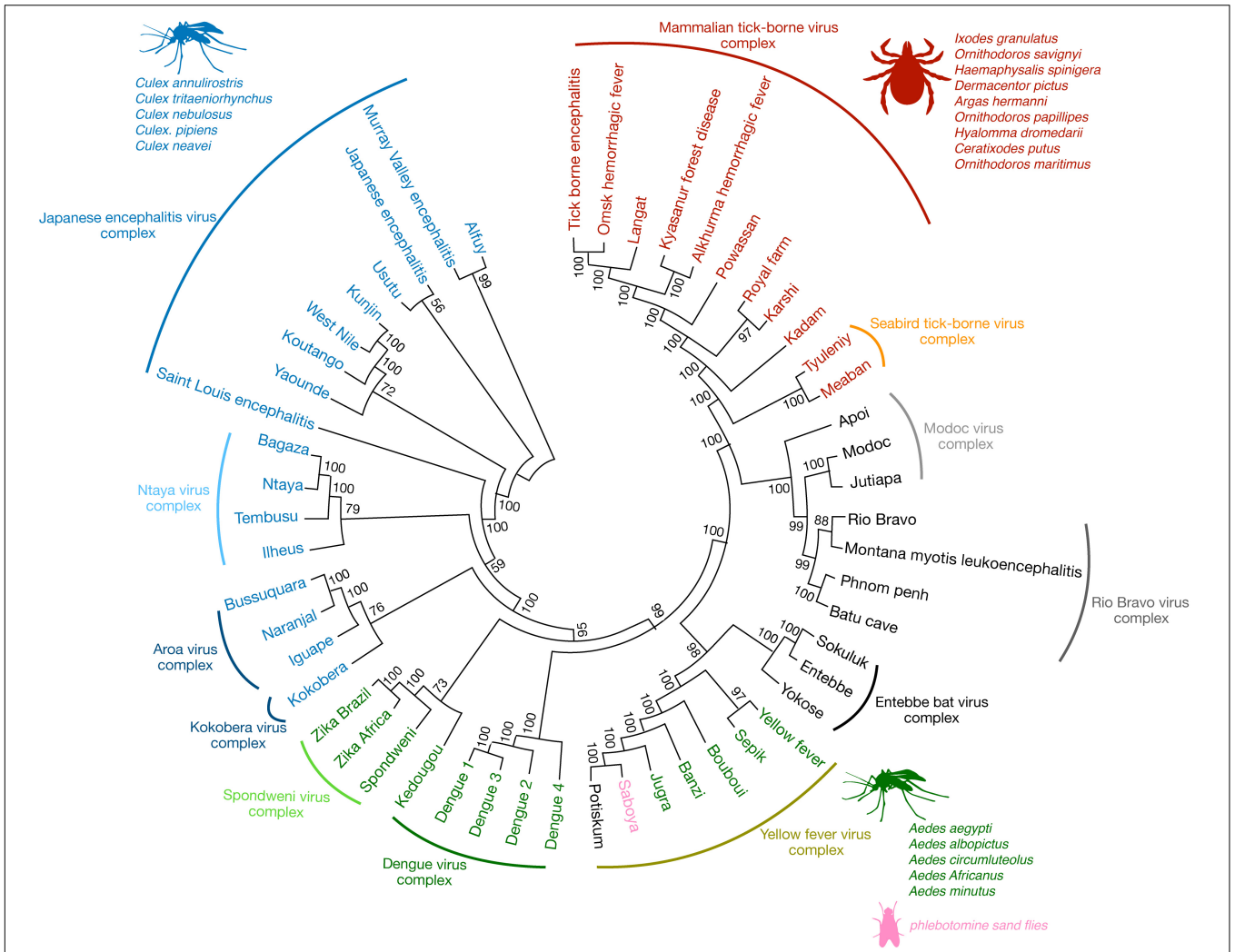

FIGURE 1 | The antigenic relationships among flaviviruses. Phylogenetic analysis demonstrates that flaviviruses cluster not just antigenically but also group according to their known transmission vectors. Some of the most significant flaviviral human pathogens belong to the JEV, Spondweni, DENV, YFV, and mammalian TBV serocomplexes, respectively (arched lines cover viruses of the same serocomplex). Some of their most common vectors are also listed, such as the mosquito species *Culex* (blue) and *Aedes* (green) and various species of ticks (red). Other flaviviruses have no known vector, for example, viruses of the Modoc, Rio Bravo, and Entebbe bat virus complex (black). Among the *Aedes* mosquito-borne viruses of the YFV serocomplex, Saboya virus (pink) has been successfully isolated from the phlebotomine sand flies (85). Phylogenetic analysis was conducted using molecular evolutionary genetic analysis (MEGA-7) software (86). The full-length polyprotein amino acid sequences from various flaviviruses were obtained from the NCBI database and pairwise aligned using Clustal W. The phylogenetic tree was constructed by using the maximum likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (87). The consensus tree representing 200 bootstrap is presented (88). Branches that were reproduced in less than 50% bootstrap replicates are collapsed. The nodes show bootstrap support values from replicates.

transmit through arthropods and distributed in to two groups belonging to the family *Togaviridae* (5). Group A comprised of arthropod-borne viruses such as chikungunya and sindbis (now in the genus alphavirus) and Group B comprised of viruses such as YFV and DENV (now in the genus flavivirus, and the subjects of this review). Because of the distinct antigenic characteristics of flaviviruses, they were later classified in to the new genus, flavivirus of the family *Flaviviridae* (6). The first arthropod-borne virus cross-reactivity was observed in complement fixation tests (7), which allows a complement reaction to occur on the surface of red blood cells (RBCs) when serum is added in the presence of a known antigen. Later, the hemagglutination inhibition assay, involving inhibition of virus-induced hemagglutination (or aggregation of RBCs) in the presence of serum was used to describe flavivirus cross-reactivity (8). Further, serological studies utilizing virus-neutralizing tests have strengthened the concept of flavivirus cross-reactivity and segregated flaviviruses that are mosquito-borne, tick-borne, and those with no known arthropod vectors (5, 9). The antigenic similarities between flaviviruses are a secondary attribute that emerges owing to their genetic similarities. As a result, infection with one flavivirus results in both species-specific and flavivirus cross-reactive antibodies. The majority of flaviviruses that are relevant to human disease were organized into 8 serocomplexes

plus 17 independent viruses that were not antigenically similar enough to warrant inclusion in a serocomplex (9) (**Figure 1**). Serocomplexes were defined by the ability of polyclonal post-immune sera against one flavivirus to neutralize others (10). Using DENV as an example, there are 4 serotypes of DENV (DENV1-4), which induce antibodies that are able to cross-neutralize each other to a certain degree, especially at high concentrations, in spite of those antibodies being insufficient to provide efficient neutralization and protection from secondary heterologous infections *in vivo* (10). In contrast, DENV-immune sera were unable to neutralize ZIKV, even though the serology indicated a relationship by another serological method [e.g. Enzyme-linked immunosorbent assay (ELISA)], supporting its close relationship to DENV but indicating that it falls into an independent serocomplex (11, 12). First described using human sera, these flavivirus cross-reactive immune responses appear to be consistent for multiple mammalian species, including rodents and non-human primates (13–15). During the acute phase of infection and disease, flavivirus cross-neutralizing antibodies can be induced, but these are usually not durable and cross-neutralization is not retained following a few months (12). Those exposed to multiple flaviviruses may also generate responses more difficult to decipher and which cross-neutralize viruses from distantly related serocomplexes (16, 17).

With the global spread of flavivirus vectors, increased human mobility, and increased vaccine coverage against flaviviruses, we are not only concerned with how pre-existing immunity could affect a heterologous challenge with a new virus from the same serocomplex (e.g. DENV2 infection followed by DENV1 infection) but also how immunity is influenced by sequential exposure to multiple flaviviruses from differing serocomplexes. Notwithstanding the potential of anti-flavivirus memory immune responses to influence subsequent infections, the high degree of immune cross-reactivity to flaviviruses makes infections, and prior exposures difficult to definitively identify when virologic confirmation is not possible (18).

FUNCTIONAL IMMUNE OUTCOMES OF SEQUENTIAL FLAVIVIRUS INFECTIONS

As humans have become more likely to experience more than one flavivirus infection during a lifetime, there is a need to understand how pre-existing immunity to a flavivirus impacts subsequent flavivirus infection outcomes. Early studies in humans exploring the nature of immune protection against flaviviruses observed that functional immune responses (virus neutralization) and the course of infection were modulated in the context of pre-existing flavivirus cross-reactive immunity (19, 20). While human studies have been largely correlation-based, studies using animal models have provided more definitive functional disease outcomes. For example, immunity to JEV and SLEV was protective against lethal WNV challenge in a hamster model (15). Similar cross-protection was observed when mice were immunized with Usutu virus and challenged with WNV (21). Within the same serocomplex, prior exposure to Kunjin or Murray Valley encephalitis (MVEV) viruses in pigs was also protective against JEV challenge (22). These studies suggest that cross-reactive immunity may be protective within the JEV serocomplex. In contrast, in a human DENV challenge study, immunity to DENV only provided lasting protection against a homologous DENV serotype (10, 19). Within the YFV complex, primary infection of rhesus macaques with Wesselsbron virus (from the YFV serocomplex) was protective against YFV challenge (13) and, more recently, examining the possibility of cross-protection against viruses in differing serocomplexes, JEV vaccination was shown to provide cross-protection against DENV and to increase the kinetics of the development of neutralizing antibody responses (14). Certain DENV or ZIKV-specific human monoclonal antibodies also can protect against a Spondweni virus challenge in immune compromised mice (23). However, primary infection with WNV (from the JEV serocomplex) or Banzi virus (from the YFV complex) failed to provide any protection against YFV challenge (13). The differences between cross-protection versus pathology have been more controversial in the context of how pre-existing immunity to DENV influences subsequent ZIKV infection. While some studies in immune compromised mice have suggested that DENV T cell immunity can be protective against ZIKV infection in adult mice (24), other studies in STAT2-KO mice, with impaired immunity, showed prior DENV immunity can enhance infection in adult mice and infection and fetal demise in pregnant mice (25, 26). In immune competent mice, pre-existing DENV immunity can enhance the development of a microcephaly phenotype in fetuses (27, 28), which is a key characteristic pathology of disease. These studies suggest that various factors determine cross-protection, some of which are discussed below.

FLAVIVIRUS CROSS-REACTIVE T CELL RESPONSES

Various subsets of T cells are essential for efficient infection clearance and for the development of robust antibody responses against flaviviruses. Flavivirus reactive T cell epitopes have been identified in both viral structural and non-structural proteins and for CD8 and CD4 T cells (14, 29). Often, we expect that the T cell epitope must be 100% conserved to induce recall of a memory T cell from a previous infection; however, similar epitopes with only minor substitutions often are able to activate T cells as well although with potentially differing degrees of responses (30, 31). Early studies using murine T lymphocyte clones demonstrated T cell cross-reactivity similar to that observed using antibody neutralization tests (32, 33). For example, T cell clones specific to Kunjin or WNV showed cross-reactivity with MVEV or vice versa (32), suggesting T cell cross-reactivity within the JEV serocomplex. T cell cross-reactivity between two different serocomplexes has also been observed. CTL clones specific for DENV reacted and proliferated against Kunjin (JEV serocomplex), a virus from different serocomplex than DENV (34). Similarly, DENV or ZIKV induced CD8

T cell responses are also noted to be highly cross-reactive against each other (35). While the majority of flavivirus cross-reactive CD8 T cells are directed against viral non-structural proteins, studies using chimeric viruses have identified cross-reactive CD8 T cell epitopes that are present in the viral envelope protein (36). This suggests that both conserved non-structural proteins and variable structural proteins contribute toward the development of the flavivirus cross-reactive CD8 T cell repertoire. Interestingly, for human T cells, prior exposure to DENV skewed the immunodominance of ZIKV-specific T cells toward non-structural proteins (35), yet these cross-activated T cells retained their *ex vivo* functionality (37). This cross-reactivity may be beneficial for clearing a secondary infection since it has been shown that a vaccine utilizing non-structural epitopes of DENV can protect against DENV infection in mice (38). Still, the broad question of whether flavivirus cross-reactive CD8 T cells are protective or pathological in nature remains to be resolved. Indeed, within the DENV serocomplex, certain cross-reactive CD8 T cell epitopes have been associated with severe disease in humans (39, 40), illustrating the potential of cross-reactivity to reduce the efficiency of an antigen-specific response during a heterologous secondary challenge (10), a well-known phenomenon termed "original antigenic sin" (**Figure 2**).

CD4 T cells also have the potential to be cross-reactive and unique clones have been shown to have *ex vivo* stimulation in response to various flaviviral antigens (14, 41, 42). More recently, the functional role of serocomplex cross-reactive CD4 T cells has been identified, where they were shown to be associated with improved viral clearance during secondary serocomplex heterologous infection (14). Genetic similarity appeared to be a factor in cross-protection in this context, where JEV provided better CD4-dependent cross-protection against DENV than the more distantly related YFV in the mouse model (14). Importantly, CD4 T follicular helper (Tfh) cells with an effector memory phenotype were shown to be critical for efficient memory recall and in improving the quality of antibody responses, providing a mechanism of cross-protection (14). In humans vaccinated against JEV, cross-reactive T effector memory cells were also identified, which could be activated to proliferate and produce interferon-gamma in response to antigens from all of the heterologous viruses tested including DENV, YFV, and ZIKV (14). During heterologous DENV infections, CD4 effector memory T cells that have a T_{EMRA} phenotype, which is cytotoxic in nature, have also been identified (43, 44) and these may be present and recalled, in addition to cross-reactive Tfh cells, during heterologous flavivirus infection. It is probable that flavivirus cross-reactive CD4 T_{EMRA} cells are also present in individuals exposed to varied flavivirus infections or vaccines but their frequency may be very low. However, the antigen presenting molecule, MHC-II is highly polymorphic in humans and therefore flavivirus CD4 T cells responses can be restricted to certain HLA-types making identified cross-reactive epitopes likely relevant for only those with the given HLA-types (10). Virion structure also determines cross-reactive CD4 T cell immunodominance, as certain structural protein conformations can provide immunodominant epitopes that are conserved structurally but not genetically among flaviviruses (45, 46).

FLAVIVIRUS CROSS-REACTIVE ANTIBODY RESPONSES

The surface glycoprotein of a flavivirus consists of type-specific and serocomplex cross-reactive epitopes, owing to which the antibodies evoked are both type-specific and cross-reactive in nature (47–49). Structural determinants such as the conformation of epitopes and the presence of flavivirus conserved residues on the surface of the viral envelope (E) protein determine antibody binding and neutralization properties. For instance antigenic epitopes present on the surface of E protein, which consists of domains I, II, and III, are both linear and quaternary and immune activation to E generates antibodies that are largely neutralizing against the virus of same type (50–52). In the context of immune responses to E protein, antibodies that are neutralizing are better generated by the quaternary structures of E protein dimers, compared to the monomeric form of the protein (53). Moreover some E dimer epitopes are conserved across related flaviviruses and evoke antibodies that are cross-neutralizing to flaviviruses of differing serocomplexes, for example antibodies that can neutralize both DENV and ZIKV (54, 55). However, antibodies targeting the E protein fusion loop epitopes, which are conserved across various flaviviruses are also broadly cross-reactive but studies so far have indicated that they are poorly neutralizing (51). These weakly neutralizing cross-reactive antibodies may influence the course of subsequent flavivirus infection, as discussed below.

Both antibody specificity and concentration can govern the ability of antibodies to neutralize or enhance the uptake of virus by Fc-receptor bearing cells (56). In some cases, individual monoclonal antibodies that are capable of cross-neutralization may be identified from an otherwise sub-neutralizing pool (57). Conversely, weakly neutralizing antibodies often can lead to neutralization at high concentrations as was shown with WNV (58) which may factor in to the period of relative resistance to similar viral infections in the months immediately following infection (56). As a result of the decay of antibody in serum in the years following vaccination or natural infection, subsequent functional responses to new flaviviruses can be temporal (56).

There are antigenic relationships amongst flaviviruses that are rarely functionally tested *in vivo* in humans due to the infrequency of the two sequential infections. For example, antibody cross-reactivity to Yokose virus (from the Entebbe bat virus serocomplex) is also observed in patients' sera infected or vaccinated with DENV or YFV (59). Yet, other sequential flavivirus challenges are much more common, such as the high probability of re-exposure to multiple serotypes of DENV or sequential exposures to DENV and ZIKV (60, 61). Much of our understanding of how flavivirus cross-reactive antibodies influence subsequent exposures to flaviviruses has been obtained in models of sequential DENV infections, where antibody-dependent enhancement of infection (ADE) by a heterologous serotype is consistently observed (10). This results from the binding of virus that is complexed with sub-neutralizing antibodies to the Fc receptors of immune cells including dendritic cells, monocyte/macrophages and mast cells (10). In this context, opsonization of virus can lead to enhanced infection in the

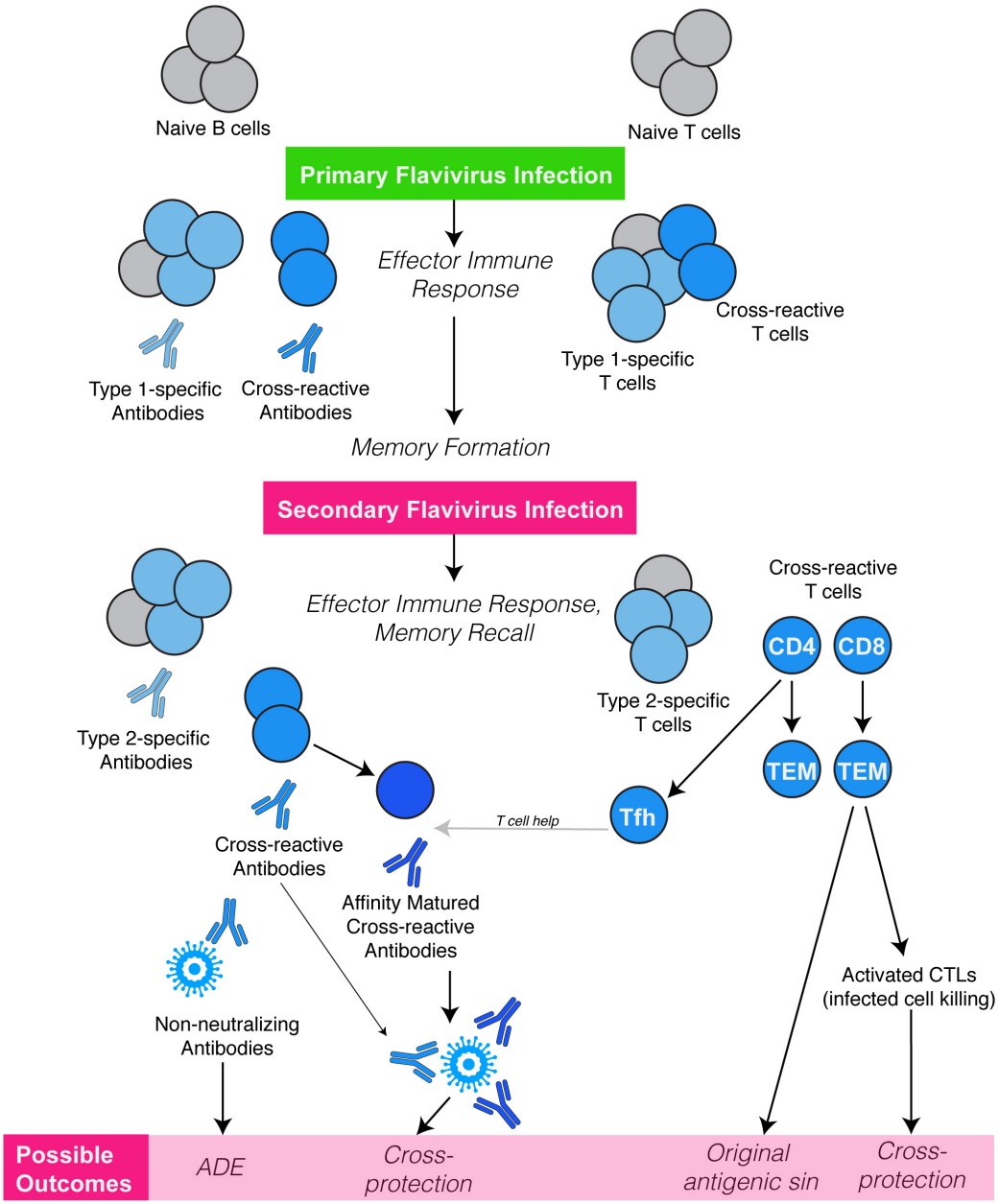

FIGURE 2 | Flavivirus cross-reactive cellular immune responses. Infection or vaccination against a flavivirus (Type-1) results in a primary immune response dominated by generation of Type-specific antibodies and T cell responses, followed by a memory formation. Owing to their antigenic relatedness, flavivirus cross-reactive antibodies and T cells (CD4 & CD8) are also generated. A secondary challenge with a second flavivirus (Type-2) can potentially reactivate cross-reactive memory T cells, those, which have higher specificity for Type-1 than for Type-2 flavivirus. These weakly cross-reactive memory T cells may outcompete naive T cells that would be more specific for Type-2, resulting in T cell original antigenic sin. However, memory T cells could also provide cross-protection directly by acquiring CTL function resulting in enhanced killing of virus-infected cells. Importantly cross-reactive CD4 Tfh cells can be recalled in the lymph node germinal centers, providing help to B cells, and improving both affinity and avidity of antibodies that are cross-reactive and neutralizing. Flavivirus cross-reactive antibodies also interact in different ways during a secondary flavivirus infection. After primary infection, high affinity and Type-specific antibodies are produced, which can neutralize virus when present at optimal concentrations. However, during secondary heterologous flavivirus infection, pre-existing cross-reactive, sub-neutralizing antibodies may lead to opsonization of virus particles and enhanced uptake by various immune cells such as monocytes via Fc receptors, resulting in increased virus replication, a phenomenon termed antibody-dependent enhancement (ADE).

immune cell types, resulting in both increased virus production and heightened production of pro-inflammatory mediators (10). Aside from opsonization, antibodies can also trigger alternate immune activation pathways such as Fc receptor cross-linking or antibody-dependent cellular cytotoxicity (ADCC) (10). During secondary heterologous exposures, mast cells activation is

observed downstream of IgG or IgE cross-linking upon virus binding (62, 63). NK cell mediated ADCC also can trigger release of pro-inflammatory and cytotoxic mediators (64). ADE occurs not only for DENV, but for other combinations of viruses from differing serocomplexes, such as DENV and ZIKV (65). Indeed, ADE of the attenuated YFV strain 17D was shown to occur *in vivo* in humans, depending on JEV vaccine-induced antibodies from a prior immunization (66). However, Fc receptor activation and binding by virus-immune complexes may not always be detrimental to the host. For example, prior vaccination to JEV was shown to enhance uptake of YFV vaccine (strain 17D) by antigen presenting cells in mouse lymph nodes, leading to increased immunogenicity of the attenuated YFV vaccine (66) and suggesting a potential utility to ADE when it improves adaptive immune responses.

Phylogenetic analyses suggest a close relationship between DENV and ZIKV (67) and studies testing human anti-DENV sera demonstrated a high degree of cross-reactivity (11). As discussed, despite this high cross-reactivity, DENV-specific antibodies fail to cross-neutralize ZIKV (12), but may lead to opsonization of ZIKV viral particles (65). Not only was this shown to occur in conventional antigen presenting cells, dependent on the Fc gamma receptors, DENV antibodies can also enhance uptake of ZIKV in the syncytiotrophoblasts, and fetal endothelial cells of the placenta in a mechanism dependent on the fetal neonatal Fc Receptor, FcRN (27). In support of the role of antibody dependent enhancement of ZIKV pathology *in vivo* in humans, mothers with antibodies that were highly enhancing to ZIKV were shown to have fetuses (or children) with more severe microcephaly phenotypes (68). This pathological influence of DENV immunity during subsequent ZIKV infection may be specific for the context of pregnancy, since epidemiologic studies in humans suggest that ZIKV infection rates may be reduced in DENV immune non-pregnant individuals (69).

Pre-existing immunity also has the potential to improve cross-reactive antibody responses that are developed during a heterologous flavivirus infection. When JEV immunity led to faster induction of neutralizing antibodies to DENV, adoptive transfer studies showed that it was based on recall of a heterologous memory response and increased germinal center activity in lymph nodes, resulting in gains in antibody avidity and neutralization against DENV in JEV-exposed animals (14). However, there are also indications that the quality of antibodies can be impeded by prior flavivirus immunity. For example, in one study, individuals pre-vaccinated against YFV were shown to have a lower ratios of neutralizing to ELISA antibody titers (70), emphasizing that the quality of immune responses and not only the magnitude are important in determining the potential of cross-reactive immunity to induce protection versus pathology (**Figure 2**).

IMPLICATIONS FOR RATIONAL FLAVIVIRUS VACCINE DESIGN

Herd immunity needs to be maintained to keep the population protected from certain flaviviruses for which vaccines are available. YFV is an example of this need since outbreaks of YFV in South America and Africa have occurred in recent years coinciding with reductions in vaccine coverage (71, 72). The success of the YFV vaccine has largely been attributed to its safety and effectiveness as an attenuated vaccine that induces immune activation of multiple pathways including innate responses, and effective T cell and antibody responses (73). A vaccine does not need to provide 100% of individual's life-long durable protection to be effective, but the YFV vaccine often does. For this reason, it has been used as the "backbone" for other vaccines including the Sanofi Pasteur DENV vaccine, Dengvaxia (74). This means that the non-structural proteins of the 17D YFV vaccine were used to construct chimeric viruses with the structural proteins of each of the DENV1-4 viruses (74). Aside from its validated safety, the YFV backbone was also chosen for this DENV vaccination strategy because the replication rates could be closely matched for all serotypes, promoting similar antigen persistence *in vivo* (74). This was to counteract the problem that was discovered early on in DENV vaccine design, where one or two of the 4 serotypes replicated much more efficiently *in vivo*, leading to immune dominance and poor coverage against multiple serotypes (75). Dengvaxia (76) has been licensed in several countries and although the effectiveness differs between serotypes it is not clear if this is due to residual differences in antigen persistence between chimeric vaccine strains or due to the influence of prior DENV immunity present in the various populations where the vaccine was tested. More pressing, in spite of the moderate and acceptable levels of efficacy shown, some safety concerns were raised, including that children, which were likely a surrogate for flavivirus-naïve individuals since the vaccine was tested in hyperendemic regions, were more likely to require hospitalization (77). Since then, it has been surmised that an ADE-like response may be occurring in certain vaccinated individuals having breakthrough cases, which is induced by the antibodies to the vaccine (78). It is also possible that the mismatch of the virus non-structural proteins to DENV is a contributing factor to the development of breakthrough cases in vaccinated individuals (10). Supporting this, T cell responses in YFV vaccinated mice were not efficiently recalled in DENV stimulated T cells and YFV-induced CD4 T cell responses showed little cross-reactivity for DENV epitopes (14). Human cross-reactive T cell responses between YFV and DENV have been detected (14). Alternate backbones have been used for DENV vaccine strategies, including the JEV backbone, which was protective in mouse models (79) and a conserved DENV backbone, itself (80). The Takeda vaccine, for example adopted this strategy, using a DENV2 backbone with DENV1-4 structural proteins (80, 81). This strategy has the potential to provide increased recall of vaccine-induced T cell responses during natural infection with DENV viruses. Recently a clinical trial demonstrated efficacy of the Takeda vaccine in humans in a dengue-endemic region as well (82), although the long-term effects of vaccination and the persistence of protection will need to be monitored alongside the potential of any breakthrough severe disease. Importantly these vaccines illustrate how homologous and heterologous immunity to flaviviruses can change the protective capacity of a vaccine and potentially

even influence its safety. Although we are concerned with the potential of cross-reactive immunity to induce pathologies following vaccination against flaviviruses, the indications that cross-protective immunity can be induced also highlight the potential of rationale design of cross-protective vaccines that are effective against multiple flaviviruses in the future.

OUTLOOK

In spite of many recent advances toward flavivirus directed vaccines, the world wide burden of flaviviruses is actually increasing. The potential of cross-reactive immunity to influence the infection outcomes and the fact that the immune profile of individuals can change over time, being different in the months, versus years, versus decades following infection, emphasizes the need to continue studying how cross-reactive immunity works and influences infection outcomes. Compounding this is the issue of flavivirus emergence. The flavivirus genus contains diverse viruses that are present in the environment and hidden in unexplored reservoirs. The recent emergence of WNV in North America in 1999 (83), ZIKV in the South Pacific in 2013-2014 (84) and South America in 2015-16 (61) and resurgence of YFV (72), in spite of a highly effective vaccine, and growing vaccine coverage for DENV emphasizes the need to consider how cross-reactive immunity will influence infection by those flaviviruses we expect and also those that don't have a significant burden in humans at this time.

AUTHOR CONTRIBUTIONS

AS and AR conducted literature search and analysis, and designed and wrote the manuscript.

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frontiers in Immunology

REVIEW
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The Case for Exploiting Cross-Species Epitopes in Malaria Vaccine Design

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The infection dynamics between different species of *Plasmodium* that infect the same human host can both suppress and exacerbate disease. This could arise from inter-parasite interactions, such as competition, from immune regulation, or both. The occurrence of protective, cross-species (heterologous) immunity is an unlikely event, especially considering that strain-transcending immunity within a species is only partial despite lifelong exposure to that species. Here we review the literature in humans and animal models to identify the contexts where heterologous immunity can arise, and which antigens may be involved. From the perspective of vaccine design, understanding the mechanisms by which exposure to an antigen from one species can elicit a protective response to another species offers an alternative strategy to conventional approaches that focus on immunodominant antigens within a single species. The underlying hypothesis is that certain epitopes are conserved across evolution, in sequence or in structure, and shared in antigens from different species. Vaccines that focus on conserved epitopes may overcome the challenges posed by polymorphic immunodominant antigens; but to uncover these epitopes requires approaches that consider the evolutionary history of protein families across species. The key question for vaccinologists will be whether vaccines that express these epitopes can elicit immune responses that are functional and contribute to protection against *Plasmodium* parasites.

Keywords: malaria, *Plasmodium*, heterologous, cross-species, immunity, vaccines, epitopes

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INTRODUCTION

A malaria vaccine would have a tremendous impact on vulnerable populations, with the potential to save nearly half a million lives annually and prevent over 200 million cases (1). Yet the development of an efficacious vaccine remains elusive. One of the biggest challenges facing malaria vaccine development is the complex life cycle of the parasite (**Figure 1**). The sporozoite form of the parasite invades hepatocytes in the liver, undergoes schizogony, and then enters the blood stage. In the blood, some of the parasites differentiate to form gametocytes that can be taken up by mosquitoes during a blood meal, resulting in onward parasite transmission. The challenge to vaccinologists

is that the parasite expresses antigens that are largely stage-specific during its lifecycle and no single defining vaccine target or even whole organism vaccine can protect against all stages. Despite this, there are multiple opportunities for vaccines to interrupt the parasite life cycle (2). A vaccine that prevents sporozoite colonization of hepatocytes could protect individuals from *Plasmodium* infection, while a vaccine targeting the blood stage could curb the clinical manifestation of disease, and a gametocyte-targeting vaccine could block transmission to mosquitoes.

Of the six species of *Plasmodium* that infect humans, *P. falciparum* causes the greatest mortality and morbidity worldwide (1). In high transmission settings, millions of young children are at risk of dying from severe falciparum malaria until they acquire immunity to severe disease later in childhood. As such, current vaccine efforts are largely focused on *P. falciparum*. Only one licensed vaccine exists, RTS,S, and this vaccine is currently undergoing pilot roll-out in several African countries. The target of RTS,S is the surface circumsporozoite surface protein (CSP) that is expressed on the surface of *P. falciparum* sporozoites (3). While this vaccine aims to prevent liver stage infection, the results from earlier vaccine trials suggest good immunity in the first six months but then a significant waning of immunity over time, resulting in poor long-term vaccine efficacy (3). This is likely due in part to the low dose of sporozoites inoculated by mosquitoes that fails to reactivate memory B-cells, combined with antigenic polymorphisms in the T-cell epitopes of the CSP (4). Other vaccines target *P. falciparum* blood stage antigens and they also face significant challenges, primarily due to antigenic polymorphisms that reduce the efficacy of allele-specific vaccines against natural infections (5).

The limitations of current experimental vaccines may reflect a shortcoming in the traditional approach to antigen discovery (6). Candidates, particularly blood stage antigens, are often identified as targets of neutralizing antibodies in immune sera; but the corollary is that this strategy selects for immunodominant epitopes that are under strong immune selection, and consequently, are highly polymorphic. Incorporating conserved and cryptic epitopes (epitopes not normally exposed to the immune system) into vaccines may overcome these challenges.

Here we consider whether epitopes conserved across species can be exploited in vaccine design. This idea may seem heretical given the absence of sterile immunity following lifelong exposure to a single species, and our understanding that the immune response to malaria is largely considered strain-specific. In fact, cross-species immunity has doubtlessly been selected *against* due to the co-circulation of multiple *Plasmodium* species competing for the same human host. Competition between parasites likely resulted in the evolution of different virulence and life cycle strategies as a form of mutual adaptation, and within these species-specific adaptations arose antigenic diversity in virulence genes of that parasite. Nevertheless, the shared evolutionary history among the six species of *Plasmodium* purports that many proteins will be homologous in origin, with common structures and/or functions. As such, it is likely that there are subdominant or even immunologically cryptic epitopes that remain conserved across multiple species. As a vaccine strategy, this presents an opportunity to direct the immune response against these conserved epitopes and exploit them in a cross-species malaria vaccine.

In this review, we discuss the evidence for immunological cross-reactivity between *Plasmodium* species and the rationale for considering a cross-species vaccine approach. We define heterologous immunity and cross-reactivity as immunological interactions between two different *Plasmodium* species and not between two strains of the same species. We first consider the clinical outcomes of natural infection in areas co-endemic for multiple species, deliberate human infection studies, and infections in animal models. Next we describe the parasite-specific immune responses to different species of *Plasmodium* and the antigens that may mediate cross-species immunity. Lastly, we provide a rationale for mapping conserved epitopes in antigens from different species and developing these epitopes as vaccine candidates.

OBSERVATIONS FROM NATURALLY EXPOSED POPULATIONS

Interactions between different species of *Plasmodium* are evident from a number of epidemiological studies of naturally exposed populations [reviewed in (7, 8)]. These are often reported as negative interactions, where co-infection with two species exacerbated disease (7), or provide no evidence of interaction at all - infection with one species had no demonstrable impact on the risk or severity of infection from another species (7). Yet concurrent studies from South Asia and Oceania gave rise to findings in support of cross-species immunity (9–11). In particular, there is compelling data that infection with *P. vivax* confers a degree of clinical protection against *P. falciparum*. This was observed in a prospective study in Sri Lanka, where the severity of symptoms from *P. falciparum* infection was lessened following a *P. vivax* infection, inferred as "clinical tolerance" to the more virulent species (9). Further support for this phenomenon was garnered from cross-sectional and longitudinal studies in Vanuatu where the incidence of severe malaria (severe anemia and cerebral malaria) was much lower than expected for an area hyperendemic for *P. falciparum* and *P. vivax* (10). The authors proposed that cross-species immunity may contribute to clinical protection and impact the infection dynamics of these two species (12). Subsequent data from a large-scale prospective analysis of health-center morbidity in Papua New Guinea provided further evidence that *P. vivax* infection was associated with clinical protection against *P. falciparum* disease (11). In all of these studies, *P. falciparum* never protected against *P. vivax* infection.

The hypothesis that one species could suppress the pathogenicity of another (7) could also account for other unusual epidemiological observations from co-endemic areas. For example, distinct seasonal patterns characterized the incidence of *P. falciparum* and *P. vivax* in Vanuatu (13) and between *P. falciparum* and *P. malariae* in Nigeria (14), where each species was dominant at different times of the year and appeared to alter

the infection dynamics of the other. Inter-species suppression of infection may also explain the recurrence of latent *P. vivax* or *P. malariae* following treatment of *P. falciparum* infections (15), and the low frequency of mixed infections in populations where multiple species co-exist (16, 17). In fact, this led to the suggestion by Cohen (16) that, "*If heterologous immunity can indeed greatly reduce the prevalence of mixed infections, as is claimed, then a malaria vaccine need not be specific to each of the species, strains, or antigenic variants of Plasmodium in order to be effective.*"

In none of these studies was there evidence that prior infection with one species reduced the risk of subsequent infection with another species. This is consistent with the lack of sterile immunity to any species of malaria, even against different strains within the same species. Rather, the evidence from these population-based studies suggests that the interactions among species may occasionally be protective and reduce the clinical course of disease. Even this cautious interpretation is subject to challenge by the many confounding factors that plague these types of epidemiological studies. It is very difficult to follow precisely the course of infection in individuals, even in longitudinal studies. This limitation is particularly apparent in light of the high frequency of submicroscopic infections revealed in more recent studies using molecular diagnostics (18). We cannot exclude persistent, submicroscopic infections of one species that could impact interpretation of these data. Alternative explanations to cross-species immunity have also been raised, including non-specific antiparasitic effects (19), ecological competition between parasites for the same mosquito host, and density-dependent mechanisms such as competition for red blood cells and nutrients within the human host (7, 16, 20). Given these limitations, we turn to studies with controlled infections in humans and laboratory animals to assess the validity of cross-species immunity.

EXPERIMENTAL HUMAN INFECTIONS

One of the earliest studies to deliberately infect human volunteers with *P. vivax* and *P. falciparum* was from the 1930s (21). Eight volunteers were infected with either *P. vivax* or *P. falciparum* from the bite of an infected mosquito then infected with the heterologous parasite either during the incubation period, the clinical phase, or following a recent infection with the first parasite. In the majority of these cases, the second infection was established, with no evidence of sterile immunity in these volunteers. Yet there was no discussion of whether the severity of symptoms during the second infection was affected by the primary infection.

The subsequent era of experimental human infections from 1940 to 1963 centered on malaria therapy treatments of patients with neurosyphilis. Treatment often resulted in multiple sequential infections with homologous or heterologous species, especially if the first treatment did not meet the therapeutic goals or due to limited availability of mosquitoes and patients infected with a particular species as a source of parasites for treatment. This may confound the interpretation of the results when comparing sequential infections to mono-infections since the reason for treatment failure is not known. Furthermore, homologous protection in control subjects was not always assessed. Despite this inherent variability, the malaria therapy cases yielded a wealth of data on the outcomes of infection with different species.

One of the earliest comprehensive reviews of these cases evaluated the effects of a primary malaria infection with *P. falciparum, P. ovale, P. malariae,* or *P. vivax* on patients re-infected with either the same or a different species (22). Upon homologous re-infection, the severity of the subsequent infection was significantly reduced but heterologous re-infections gave variable results. The outcome depended on the combination and order of species for the primary and secondary infections. In fifteen patients with a *P. vivax* infection followed by a *P. falciparum* infection, no effect on the second infection was observed when peak asexual parasitemia, gametocytemia and fever episodes were compared to single infections in malaria-naïve individuals. Similarly, there was no effect of a *P. malariae* infection on a subsequent *P. falciparum* infection (n = 6). However, previous infection with *P. vivax* led to lower parasite densities and fewer fever episodes during a subsequent *P. ovale* infection in 15 patients, compared to naïve individuals. When the order of these infections was reversed and *P. ovale* was given first, there was no effect on fever or parasitemia during the following *P. vivax* infection; however, the *P. vivax* infections were self-limiting, and no drug treatment was required.

There was a similar effect when a *P. falciparum* infection followed a *P. ovale* infection (n = 11). In these cases, there was obvious modification of the severity of the *P. falciparum* infection resulting in a much lower proportion of patients requiring treatment and in those that did, a lower therapeutic dose was sufficient. In fact, no curative doses of drugs were needed if the *P. falciparum* infection was preceded by a *P. ovale* infection. When the order of these infections was reversed in eleven patients, there was no effect of prior *P. falciparum* infection on the subsequent *P. ovale* infections. Only a small number of patients received a *P. vivax* infection after a *P. malariae* infection, but the *P. vivax* infection in 2 of 3 patients resolved spontaneously.

A later review of different patient files from the same time period suggested some cross-reactivity of *P. falciparum* with *P. malariae*, but not with *P. vivax* or *P. ovale* (23). The frequency of *P. falciparum* hyperparasitemia ($\geq$ 10,000/μL) and fever was not affected by prior infection with *P. vivax* or *P. ovale* but was reduced when the *P. falciparum* infection was preceded by a *P. malariae* infection. This latter observation was countered in another review, concluding there was no evidence that past or current *P. malariae* infection affected *P. falciparum* asexual parasitemia; yet interestingly, there was an effect on *P. falciparum* gametocytemia (24). The authors proposed that the balance between asexual parasitemia and gametocytemia could be altered by the presence of the other species.

Collectively, the data from select human experimental studies bolster the evidence for cross-species interactions observed in the field studies, although this is clearly not a consistent occurrence. These studies further highlight the non-reciprocal nature of parasite interactions that appear to be predicated on the temporal

sequence of infection and support a mechanism of partial heterologous immunity that can limit disease severity from the secondary infection.

INFECTIONS IN EXPERIMENTAL ANIMALS

Animal models of malaria offer an analogous approach to investigate cross-species immunity in a controlled environment. Early studies that investigated interactions between *P. gallinaceum* and *P. lophurae* in chickens corroborated the findings of the various human studies that heterologous immunity can be non-reciprocal (25). Chickens infected with either *P. gallinaceum* sporozoites or blood stage parasites followed by infection with homologous parasites [as sporozoites or infected red blood cells (iRBCs)], or iRBCs of the heterologous species *P. lophurae*, exhibited marked reductions in both homologous and heterologous parasitemia. But when the order of the inoculations was reversed, weak heterologous immunity was observed and only in chickens that were hyperimmune to *P. lophurae* (following 4 or 5 infections).

There is ample evidence from mouse models that vaccination with attenuated sporozoites can elicit cross-species protection [reviewed in (26, 27)]. Mice immunized with X-irradiated *P. berghei* sporozoites were completely protected from heterologous challenge with *P. vinckei* sporozoites and immunization with irradiated *P. chabaudi* sporozoites induced sterile protection against infection with *P. berghei* (28). Furthermore, both irradiated and genetically attenuated *P. berghei* sporozoites inhibited intrahepatic development of *P. yoelii* sporozoites based on copies of parasite 18S ribosomal RNA quantified by qRT-PCR (29). Even immunization with *P. falciparum* sporozoites protected 60% of mice from a *P. berghei* infection (but not a *P. yoelii* infection) and passive transfer of IgG from these *P. falciparum* vaccinated mice protected naïve mice from a *P. berghei* sporozoite challenge (30). Similarly, chemically attenuated *P. berghei* sporozoites protected mice from challenge with *P. yoelii* sporozoites (31). In this case, cross-species protection was short-lived and did not last beyond 10 days post-immunization (31).

Likewise, blood stage murine parasites are capable of inducing cross-species immunity, which was reviewed extensively by Richie (7, 8). For instance, protection - measured as reduction in mortality - was observed when *P. berghei*-vaccinated mice were challenged with *P. yoelii* and when *P. vinckei*-vaccinated mice were challenged with *P. chabaudi* (32). Similar to Taliaferro and Taliaferro's observations (25) with *P. gallinaceum* and *P. lophurae* in chickens, heterologous immunity in mice was non-reciprocal (32). Prior infection with *P. berghei* or vaccination with formalin-fixed blood-stage parasites reduced mortality in mice from *P. yoelii* infection, but no protection was observed when the species order was reversed. Similarly, mice vaccinated with *P. vinckei* were protected from *P. chabaudi* but not the inverse. One exception was the reciprocal cross-species protection between the blood stages of *P. berghei* and *P. vinckei.* After vaccination or infection with either parasite, 40–50% of mice survived a lethal heterologous challenge with the other species. From these studies and others, the genetic background of the mouse is likely to impact cross-protection. *P. chabaudi* immunization did not protect against *P. yoelii* challenge in outbred CD-1 mice (32), whereas partial protection was observed in BALB/c mice, and complete protection in C57BL/6 and CBA mice (33). More recently, this was observed using a different vaccination scheme termed 'controlled infection immunization' where mice were immunized with one species while under doxycycline chemoprophylaxis then challenged with the heterologous species (34). C57BL/6 mice immunized with *P. chabaudi* or *P. yoelii* promoted survival following heterologous challenge with the reciprocal parasite (34). While in BALB/c mice, protection was non-reciprocal; only *P. chabaudi* immunization could protect against *P. yoelii*, mirroring the findings from the older study.

Immunity in the studies described above was defined as protection from mortality, but as McColm and Dalton discuss (32), there is evidence of significant modulation of infection between species. This clinical suppression of disease was apparent as reduced parasitemia over the course of infection and delayed mortality relative to controls. Similarly, cerebral malaria was prevented in mice with a *P. berghei* ANKA infection if they had a co-infection with *P. yoelii* (but not with *P. vinckei* or *P. berghei* NK25) (35). It is important to note that in many of these studies the effects of non-specific anti-disease factors (such as cytokines or hormones) on secondary infections are impossible to separate from specific immune responses. Non-specific immune factors in sera from mice with a malaria infection inhibited *in vitro* growth of *P. falciparum* independent of antibody levels (36). Another factor may be hepcidin, which is upregulated in response to a blood stage malaria infection and inhibits a concurrent liver stage infection irrespective of the strain or *Plasmodium* species (37).

CROSS-REACTIVE ANTIBODIES

The evidence supporting cross-species protection from human and animal studies validates efforts to explore heterologous vaccine strategies but also begs an understanding of the underlying immune mechanisms. Rather unexpectedly, insight into the immunological basis of cross-reactivity first emerged from attempts to develop species-specific diagnostic tests. In testing the specificity of a complement fixation assay for malaria diagnosis, Kingsbury detected cross-reactivity between *P. vivax* and *P. falciparum* antigens (38). Sera from 6 of 12 individuals with acute *P. vivax* infection reacted to *P. falciparum* antigens in a precipitin test, and likewise, 5 of 16 sera from patients infected with *P. falciparum* reacted to *P. vivax* antigens. However, a later paper by Mayer and Heidelberger (39) suggested that the specificity of the test was compromised by reactivity of sera with human stromata in the antigen preparations. In a different precipitin test developed by Taliaferro et al. (40, 41), sera from patients in Honduras infected with *P. vivax* reacted with antigens prepared from a *P. falciparum*-infected placenta. Surprisingly, heterologous reactions were as strong as the homologous ones.

These findings were replicated in two separate studies in Honduras with over 500 sera but not in a later study in Puerto Rico with antigens prepared in the same manner (42). The results from the Puerto Rico study were deemed inconclusive and the inconsistency attributed to the generally poor performance of the precipitin test at that time. Serological cross-reactivity was later observed against *P. falciparum* and *P. vivax* crude antigens prepared from short-term culture of parasites isolated from infected patients, but the homologous reactions were more intense than the heterologous reactions (43).

With the advent of techniques to fluorescently label antibodies, their recognition of antigens from distinct malaria species could be directly observed under the microscope with the immunofluorescence assay (IFA). In one of the first records of this method applied to the study of human malaria, fluorescently labeled immunoglobulin from a patient with a long-standing *P. vivax* infection recognized RBCs infected with the simian malaria species *P. cynomolgi* (although not *P. berghei*) (44). This finding was replicated in another study where sera from *P. vivax*-infected patients (n = 4) recognized thin blood smears made from monkeys infected with *P. cynomolgi* (45). Homologous parasites were recognized much more strongly than heterologous parasites, but cross-reactivity in this case was reciprocal: serum from 5 volunteers infected with *P. cynomolgi* recognized two strains of *P. vivax* (Chesson and Venezuelan strains) by IFA using thin blood smears made from infected patients (45). Similarly, antibodies from a laboratory worker following an accidental *P. cynomolgi* infection recognized thin smears of *P. vivax* iRBCs as strongly as those infected with *P. cynomolgi* (44).

Immunological cross-reactivity between *P. vivax* and *P. falciparum* was also demonstrated with sera from naturally infected individuals (46). Sera from 9 out of 29 individuals with a *P. vivax* infection recognized *P. falciparum* iRBCs, while sera from 11 out of 21 individuals with a *P. falciparum* infection recognized *P. vivax* iRBCs. In this same study, cross-reactivity was also observed in individuals deliberately infected with *P. vivax* or *P. falciparum*. Based on the antibody titers against homologous versus heterologous iRBCs, *P. vivax* sera were more cross-reactive against *P. falciparum* iRBCs than the converse.

In Guatemala, sera from individuals naturally exposed to *P. vivax* strongly cross-reacted with asexual *P. falciparum* antigens by ELISA (20/43 positive), IFA (35/36 positive) and by immunoprecipitation assays (32/32 positive) (47). In order to rule out past *P. falciparum* infection as the source of these antibodies (despite > 99% prevalence of *P. vivax*), the sera were also tested against the *P. falciparum* CSP and heat shock protein (HSP) 70 kD-like-molecule repeat peptides by ELISA. Only 2 out of 36 sera samples recognized the PfCSP repeat peptide and 1 out of 33 sera samples recognized the *P. falciparum* HSP70 kD-like-molecule repeat peptide (48), suggesting the antibodies were truly cross-reactive. In this study, serological recognition of a HSP70 peptide was used to rule out antibodies specific to *P. falciparum* infection, but this family of proteins contains other epitopes that are shared across *Plasmodium* species (49). Given their ubiquitous nature, it is possible that these and other conserved housekeeping antigens underpinned some of the cross-reactivity discussed previously. While these may be viable targets of cross-reactive antibodies, their validity as vaccine candidates would depend on whether they elicit functional antibodies.

IFA was also useful to validate the interactions between the different species of rodent malaria and to develop a model of antigenic similarity among these parasites (50). Hyperimmune sera generated by infecting mice three times with either *P. berghei, P. yoelii, P. chabaudi,* or *P. vinckei* revealed that the four species were serologically indistinguishable by IFA. Sera from rabbits immunized with soluble antigens from these parasites gave similar results. These findings form the basis of a proposed model of antigenic conservation between the four murine malaria species whereby certain antigens are shared among all four species, some antigens are shared only between the most similar pairs of parasites and then others are specific to each species (50).

CROSS-REACTIVE T-CELLS

Cellular immunity is also likely to play a role in cross-species immunity and may underpin the protective clinical effects (reduced symptoms and disease severity) - yet there is scant data on the potential contribution of T-cells to this immune mechanism. In rodent models, antibody-independent mechanisms clearly influenced susceptibility to heterologous challenge (51). For example, B-cell deficient mice chronically infected with *P. yoelii* were resistant to lethal challenge with *P. chabaudi* (51). Cross-reactive T-cell responses are also vital to the heterologous immunity observed in murine malaria models of attenuated sporozoite vaccination. Immunization with radiation-attenuated *P. berghei* sporozoites protected 79% of mice challenged with *P. yoelii* sporozoites and immunization with *P. yoelii* sporozoites protected 63% of mice challenged with *P. berghei* sporozoites (52). Heterologous protection was dependent on $CD8^+$ T-cells whereas antibodies from immunized mice only recognized homologous, but not heterologous, sporozoites. In another study, 100% of mice immunized with genetically attenuated *P. yoelii* sporozoites were protected against *P. berghei* sporozoite challenge (53). The authors suggested that late-liver stage arresting sporozoites elicited a broadly protective $CD8^+$ T-cell response.

There are few reports of species-transcending T-cells in humans. The most definitive study showed that T-cells isolated from volunteers immunized with attenuated blood stage *P. falciparum* parasites proliferated *in vitro* in response to *P. knowlesi* iRBCs (54). Whether these T-cells have functional activity to protect against heterologous challenge is not known.

POTENTIAL VACCINE TARGETS

The data presented in this review build the case for heterologous immunity elicited by natural or deliberate infection in animals and humans. The mechanism of immunity likely involves both humoral and cellular immunity, but the antigenic determinants are unknown. To translate the observations spanning the last century into viable heterologous vaccine candidates, the conserved targets of immunity must be identified. Given that

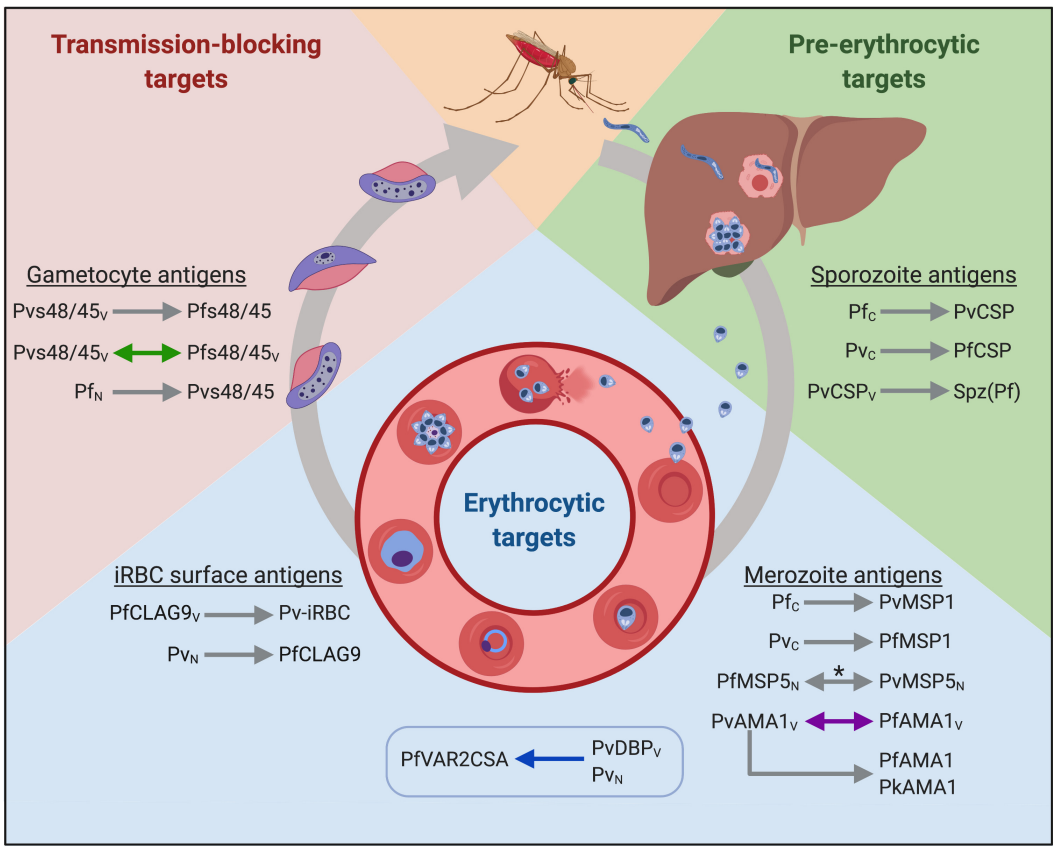

FIGURE 1 | Putative cross-species vaccine candidates at different stages of the parasite life cycle. Arrowheads indicate the direction of cross-reactivity and double arrowheads show reciprocal cross-reactivity. Gray arrows denote immunological cross-reactivity, but unknown functional activity; purple arrows denote that heterologous function was not demonstrated; blue arrows denote that heterologous function was demonstrated, and green arrows denote cross-boosting following heterologous vaccination. The box indicates heterologous cross-stage reactivity (antibodies to the merozoite antigen recognize an iRBC surface antigen). Spz(Pf) = *P. falciparum* sporozoites. Subscript letters denote route of exposure to parasite or antigen; C = Controlled human malaria infections (CHMI); V = exposure through vaccination; N = natural infection. *Antigen recognition was blocked by heterologous antigen in a subset of samples from co-exposed individuals. Created with Biorender.com.

partial protection is observed in nature and appears to be a relatively rare event, we expect that multiple vaccine candidates will be needed to target different stages of the parasite's lifecycle. Cross-species vaccines that aim to prevent infection should target sporozoite antigens to inhibit hepatocyte invasion and development, while vaccines that target blood stage antigens could prevent severe disease. Gametocyte antigens are also attractive targets to prevent the onwards transmission of malaria. Below, we review the discovery and current knowledge of potential cross-species antigens from different parasite stages. We summarized the findings from studies using human malaria parasite antigens in **Figure 1**.

Pre-erythrocytic Targets

The focus of immunity to sporozoite/liver stage infection is largely on CSP. Cross-reactive immune responses between CSP from *P. vivax* and *P. falciparum* have been reported in both naturally exposed populations (55) and in controlled human malaria infections (CHMI) (56). In populations from a region in Brazil endemic for both species, peripheral blood mononuclear cells (PBMCs) were highly responsive to stimulation with either PfCSP or PvCSP (55). Responses to both species were especially frequent in individuals recovering from a recent *P. vivax* infection; PBMCs from 35 to 54% of these individuals proliferated in response to PfCSP (55). These findings suggested that PvCSP and PfCSP might share cross-reactive T-cell epitopes, while there was no evidence of heterologous antibody responses. In contrast, deliberate infection of naïve volunteers with either *P. falciparum* or *P. vivax* by mosquito bite gave rise to heterologous antibody responses to CSP from each species (56). In both groups, 61% of volunteers had antibodies that cross-reacted with the heterologous CSP antigen. These heterologous responses were largely mediated by IgM and not IgG.

Only one study supports a role for CSP in cross-species protection, a key criterion for pursuing CSP as a heterologous vaccine target. In mice, a $CD8^+$ T-cell clone generated through vaccination with irradiated *P. yoelii* sporozoites recognized a peptide from PyCSP and the homologous peptide from *P. berghei* PbCSP (57). Adoptive transfer of this clone to naïve mice protected against homologous (*P. yoelii*) and heterologous

(*P. berghei*) sporozoite challenge. The specificity of the T-cell epitope appears to be critical for cross-species immunity. In another study, mice were immunized with attenuated *P. berghei* sporozoites and $CD8^+$ T-cells that recognized a different peptide from PbCSP were selected and transferred into naïve mice (58). These $CD8^+$ T-cells only recognized the peptide from PbCSP and not a related peptide from PyCSP that differed in sequence at three amino acid positions. Consistently, the recipient mice were only protected against a homologous challenge with *P. berghei* sporozoites, and not against heterologous challenge with *P. yoelii* sporozoites. Similar species-specific T-cell responses were observed in response to vaccination with *P. yoelii* antigens (59, 60). Mice were immunized with a T-cell epitope from PyCSP and lymph node cells isolated from these mice specifically inhibited development of *P. yoelii* liver stage schizonts *in vitro*, but not *P. berghei* schizonts (59). However, immunization with this epitope did not significantly protect mice against a homologous sporozoite challenge. In a follow-up report to this study, Rénia et al. (61) demonstrated homologous protection by passively transferring peptide-specific T-cell clones to naïve mice. They failed to observe inhibitory cross-reactivity of these T-cells against *P. berghei* sporozoites *in vitro* and therefore did not test for cross-reactivity *in vivo*.

Consistent with the lack of protection observed in some of the rodent studies, immunization of mice with *P. falciparum* PfCSP conferred no protection against heterologous challenge with *P. berghei* sporozoites (30). Similarly, immunization with B-cell epitopes from the *P. falciparum* and *P. yoelii* CSPs inhibited homologous sporozoite invasion *in vitro* but had no effect against heterologous sporozoites (62). It is perhaps not surprising that cross-species immunity is not mediated through PfCSP. The RTS,S vaccine based on PfCSP is poorly boosted by natural infection and fails to elicit robust strain-transcending immunity, with no prospects for species-transcending immunity since the CSP repeats in each species are very different (63). However, it is possible that PvCSP could prove a better candidate. Sera from mice immunized with a PvCSP vaccine candidate recognized both *P. falciparum* and *P. berghei* sporozoites by IFA (63). Immunized mice were also protected from a *P. berghei* infection initiated by the bite of an infected mosquito. As described earlier, cross-species immunity is mostly non-reciprocal and in many of the studies reviewed here, *P. vivax* confers broader cross-reactivity compared with *P. falciparum*.

Other liver stage antigens are potential targets for a cross-reactive vaccine. Sera from mice immunized with the *P. falciparum* cell-traversal protein for ookinetes and sporozoites (PfCelTOS) recognized *P. berghei* sporozoites by IFA and protected 60% of BALB/c mice from infection with *P. berghei* sporozoites (64). However, cross-species protection was not observed when PfCelTOS was expressed from a viral vector (65). The *uis3* gene represents another potential liver stage target and is conserved across human, primate and rodent *Plasmodium* species. It is actively transcribed in sporozoites but translationally repressed until the parasite infects hepatocytes (66). Mice immunized with PfUIS3 and challenged with *P. berghei* sporozoites exhibited a significant delay in the time to patent parasitemia (65). It should be noted that PbUIS3 was not specifically shown to mediate this cross-species protection and a search for predicted cross-reactive linear epitopes did not reveal peptides with high conservation between the two orthologs. PfLSA3, another sporozoite and liver stage antigen with unknown function, elicits cross-reactive antibody and cellular immune responses against rodent malaria (67, 68). PfLSA3-specific antibodies purified from hyperimmune human sera or from an immunized chimpanzee recognized *P. yoelii* sporozoites by IFA and western blot, blocked invasion of murine hepatocytes by *P. yoelii* sporozoites and protected mice from *P. yoelii* challenge in a pilot experiment (n = 4). The epitopes shared between the *P. falciparum* and *P. yoelii* proteins enable reciprocal immune recognition as antibodies from mice infected with *P. yoelii* recognized peptides from PfLSA3 yet given the absence of a PfLSA3 ortholog in *P. yoelii*, these antibodies may be targeting another related antigen or the cross-reactivity is not specific. Furthermore, this cross-reactivity is restricted to *P. yoelii,* as sera from mice infected with *P. berghei* did not cross-react with PfLSA3 and likewise, human PfLSA3 antibodies failed to recognize *P. berghei* sporozoites or block invasion of hepatocytes.

Erythrocytic Targets

The merozoite surface protein (MSP) family includes several blood stage vaccine candidates whose homology across different *Plasmodium* species may be exploited for a cross-species vaccine. For example, IgG responses from a subset of individuals in Indonesia were cross-reactive to both merozoite surface proteins PfMSP5 and PvMSP5 (69). Sera from 82 individuals with a *P. falciparum* infection, 85 individuals with a *P. vivax* infection, 85 individuals with mixed infections and 87 exposed, but asymptomatic individuals, were tested by ELISA. Of these, 107 dual-positive responders were identified that recognized both PfMSP5 and PvMSP5. Using competition ELISAs, 7 samples were identified as truly cross-reactive; in other words, recognition of MSP5 from either species could be blocked by pre-incubation with the MSP5 from the other species. Although the overall frequency of cross-reactivity to these two proteins was low (7%), these findings suggest that a vaccine that targets the cross-reactive epitopes may protect against more than one *Plasmodium* species.

MSP-1 is another viable blood stage candidate. In the CHMI study described earlier (56), 50% of volunteers infected with *P. vivax* had antibodies to PfMSP-1 while 67% of those infected with *P. falciparum* recognized PvMSP-1 on day 28 after infection. Far lower frequencies of cross-reactivity were observed in other studies. Using a multiplex bead assay, the species specificity of IgG responses to the $MSP1_{19}$ region from *P. falciparum, P. ovale, P. vivax,* and *P. malariae* was evaluated in sera from experimentally infected chimpanzees, infected individuals living in low transmission settings in Haiti and Cambodia (n = 12), and sera eluted from blood spots collected from individuals living in a high transmission setting in Mozambique (n = 20) (70). All of the antibody responses from the chimpanzees were species-specific and recognition was completely blocked by competition with the homologous protein. Only one out of 12 samples from

people living in the low transmission setting and 8 of 20 samples from people living in high transmission settings showed partial, and highly heterogeneous cross-reactivity to select other species. Cross-reactivity was mostly non-reciprocal and there were very few sera that cross-reacted with all four species of *Plasmodium*.

Another pair of orthologs that share B-cell epitopes is PfCLAG9 and PvCLAG7 (71). These proteins localize to the rhoptries and play a role in erythrocyte invasion. In this study, cross-reactive antibodies were observed in naturally exposed populations in the Brazilian Amazon and these findings were modeled in mice. Antibodies from mice immunized with PfCLAG9 peptides exhibited very similar surface staining of RBCs infected with either *P. vivax* or *P. falciparum* by IFA. The functional activity of these antibodies against the heterologous parasite was not reported.

Antigens that elicit cross-reactive antibodies against orthologous proteins from non-human and human *Plasmodium* species would be attractive vaccine candidates since their broad conservation across the evolutionary spectrum of these parasites implicates these proteins in parasite survival. Recombinant *P. falciparum* HGPXRT stimulated mouse $CD4^+$ T-cells primed with the *P. yoelii* ortholog, implying these proteins share T-cell epitopes (72). *In vivo*, mice immunized with *P. falciparum* HGPXRT controlled parasitemia and induced partial protection against a *P. yoelii* challenge. Another promising group of proteins are the merozoite-released soluble proteins (MRSPs) (73). Mice immunized with the *P. falciparum* MRSPs were protected against a blood stage *P. yoelii* infection and IgG purified from mice infected with *P. yoelii* inhibited *P. falciparum* growth and invasion *in vitro*. Furthermore, *P. falciparum* MRSPs bound to mouse erythrocytes and *P. yoelii* MRSPs bound to human erythrocytes, suggesting conservation of functionally related proteins across the two species.

Apical membrane antigen 1 (AMA1) is another viable cross-species vaccine candidate; it is an invasion protein present in many *Plasmodium* species and there is strong evidence of structural and functional conservation between orthologs (74). In fact, PvAMA1 replaced PfAMA1 in transgenic parasites without compromising parasite growth (74). Polyclonal rabbit antibodies raised against either antigen recognized the heterologous protein by western blot and stained the parasites by IFA. Further support for immunological cross-reactivity among these proteins stems from epitope mapping studies with a monoclonal antibody (mAb) raised against PvAMA1 that recognized AMA1 from *P. knowlesi, P. falciparum, P. cynomolgi* and *P. berghei* by IFA (75). Co-crystallization of the mAb with either PvAMA1 or PfAMA1 revealed striking structural similarity between the epitopes in both proteins and most of the contact residues were conserved. Interestingly, this conservation extends to the AMA1 orthologs from the other *Plasmodium* species recognized by the same mAb. It is important to note that in both of these studies on AMA1, there was evidence of antibody cross-reactivity, but these antibodies were not functional. The rabbit sera against PfAMA1 did not block RBC invasion by the *P. falciparum* transgenic strain that expressed PvAMA1 (74). Likewise, the PvAMA1 mAb recognized *P. cynomolgi* but did not block invasion by this species *in vitro* (75). This may be due to lower avidity of the PvAMA1 mAb against the heterologous antigens, as shown by surface plasmon resonance (SPR) with PfAMA1. These data provide a starting point to design a cross-species vaccine against AMA1 but emphasize the importance of defining epitopes that will yield inhibitory antibodies against the heterologous species.

Transmission-Blocking Targets

The goal of a transmission-blocking vaccine is to disrupt the life cycle of the parasite by interrupting transmission to the mosquito. A vaccine with the potential to achieve this across multiple species would be a pivotal public health tool to support malaria elimination. To the best of our knowledge, only the gametocyte antigen P48/45 has emerged as a candidate for cross-species recognition. Sera from school-aged children living in a *P. falciparum* endemic area of Zimbabwe were highly cross-reactive to the *P. vivax* homolog Pvs48/45 (76). Thirty-six of 49 (73%) samples positive for Pfs48/45 by ELISA also recognized Pvs48/45. These results were confirmed by western blot on 23 randomly selected samples. Officially, there was no *P. vivax* transmission in the area at the time of sample collection (2015), but the authors detected one low-level *P. vivax* infection out of 27 randomly selected blood samples that were tested by nested PCR, suggesting a potential caveat to these findings. Nonetheless, similar results were observed in mouse models (77). Sera from mice immunized with recombinant Pfs48/45 or Pvs48/45 recognized the heterologous proteins by ELISA and IFA. Importantly, the antibody responses against heterologous antigens were cross-boosted; for example, mice immunized once with Pfs48/45 then boosted with Pvs48/45 rapidly acquired anti-Pfs48/45 antibodies that were not present following the primary immunization. This strongly implicates that specific B-cell epitopes are conserved across the orthologous proteins. A vaccine based on these epitopes that could be boosted by natural infection with either species would be a powerful intervention against malaria.

Cross-Species Immunity to Heterologous Proteins

All the studies considered above investigated cross-reactivity between orthologous proteins yet there is evidence (although sparse) of cross-reactivity between functionally unrelated antigens from different species. We demonstrated immunological cross-reactivity between heterologous proteins in *P. vivax* and *P. falciparum* (78, 79) (**Figure 2**). Based on the unexpected finding that Colombian men and children had antibodies to the pregnancy-specific *P. falciparum* antigen VAR2CSA, we discovered that prior exposure to *P. vivax* Duffy Binding Protein (PvDBP) can give rise to antibodies that cross-react with VAR2CSA (78). We further mapped an epitope in the Duffy Binding Like (DBL) domain of PvDBP that mediates this cross-reactivity (79). Human antibodies affinity-purified against this epitope can block adhesion *in vitro* of VAR2CSA-expressing iRBCs to the placental receptor chondroitin sulfate A (CSA). The surprising aspect to this immune pathway is that PvDBP and VAR2CSA are not functionally related. Rather, they have a common homologous ancestor which gave rise to

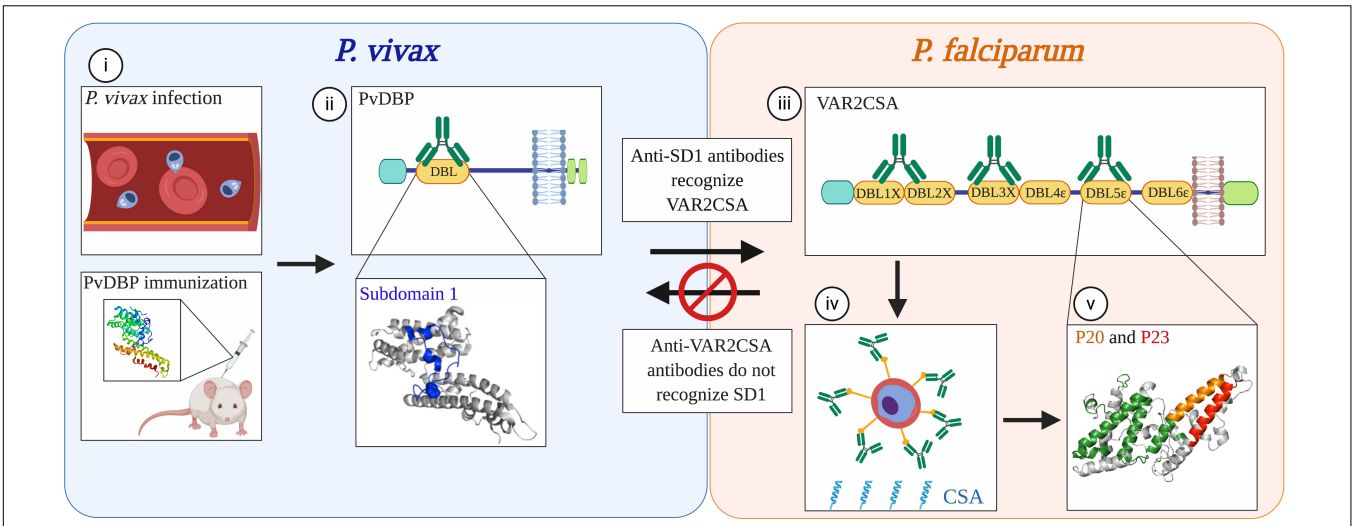

FIGURE 2 | Non-reciprocal, cross-species immunity mediated by conserved domains in functionally distinct proteins from *P. vivax* and *P. falciparum*. (i) Antibodies to the *P. vivax* merozoite protein PvDBP that arise from natural infection in humans or by vaccination with the recombinant protein in mice recognize epitopes within the DBL domain of PvDBP (ii). A subset of antibodies (green) that recognize subdomain 1 (SD1; blue) also cross-react with the DBL domains of *P. falciparum* VAR2CSA (iii), a protein that mediates sequestration of parasites in the placenta. Although PvDBP is not thought to play a role in *P. vivax* pregnancy-associated malaria, antibodies against the SD1 region of PvDBP can block *P. falciparum* parasites from binding *in vitro* to CSA, the placental ligand (iv). The recognition sites of these cross-reactive antibodies in one of the DBL domains of VAR2CSA, DBL5ε, map to two non-overlapping peptides, P20 (orange) and P23 (red) (v). These epitopes are spatially distinct from the immunodominant epitopes recognized by sera from multigravid women from Tanzania (green). Concordantly, these epitopes are cryptic; P20 and P23 are not recognized by sera from multigravid women from Uganda, nor by sera from rabbits immunized with VAR2CSA. As observed in other studies of human and mouse malaria, the immune recognition of these proteins is non-reciprocal as antibodies elicited through natural exposure to VAR2CSA in pregnant women or through immunization of animals with recombinant VAR2CSA did not recognize SD1. The cross-reactivity of antibodies to PvDBP and VAR2CSA exemplifies a mechanism of immune recognition to functionally distinct proteins in different *Plasmodium* species that is mediated by structurally conserved domains. Modified from (79). Created with Biorender.com.

conserved structural features shared among a large family of erythrocyte binding proteins (80). These structural domains are ubiquitous in human and rodent *Plasmodium* species. However, the immunogenicity of cross-reactive epitopes within these proteins is likely variable across species. In *P. vivax*, the cross-reactive epitope is subdominant, arising only in a subset of exposed individuals (79, 81). In turn, these antibodies target epitopes in VAR2CSA that are cryptic, and reciprocal immunity is not elicited by exposure to VAR2CSA in pregnancy or by vaccination (79). Cross-reactivity is therefore a rare event – but once identified, can be exploited for vaccine design.

It is even conceivable that a vaccine could be designed to confer protection across species and across parasite stages. A 60 amino acid peptide based on a cryptic epitope discovered in PfCSP elicited antibodies in mice that recognized asexual blood stages of both *P. falciparum* and *P. yoelii* by IFA and blocked *P. falciparum* merozoite invasion by 70% *in vitro* (82). Strikingly, more than 60% of mice immunized with the PfCSP peptide survived a lethal blood stage infection with *P. yoelii* (although only 2 control animals were included). The authors reported that the PfCSP anti-peptide sera recognized a 60–65 kDa parasite protein in *P. falciparum* blood stage lysates. This protein may be related to TRAP, a protein expressed during liver and blood stages that shares amino acid similarity to the PfCSP peptide sequence. Importantly, this study demonstrated that a peptide vaccine based on a cryptic epitope can focus the immune response on conserved regions of the protein, with the potential to target related antigens in other stages of the parasite life cycle.

DISCUSSION

The Rationale for a Cross-Species Vaccine

These studies revealed that individual antigens can elicit cross-reactive immune responses. However, the lack of sterilizing immunity to malaria during a lifetime of natural infection implies that a multivalent vaccine would be needed to provide cross-species protection. It is conceivable that the whole parasite vaccine approach could replicate the partial cross-species immunity observed in the studies discussed above. Parasites attenuated by irradiation, chemical treatment or genetic modification expose the immune system to a broad spectrum of antigens for that given stage, including antigens that are highly conserved across species (e.g. housekeeping antigens). If these attenuated parasites can persist in vaccinated individuals and remain metabolically active (83), this creates an opportunity for sustained antigenic stimulation of either B- or T-cells with the potential for more robust protection from future infection. In an older review of CHMI studies, only one volunteer was immunized with radiation-attenuated *P. falciparum* sporozoites and challenged with *P. vivax*. This person was not protected from

vivax infection (84). Further CHMI studies are needed to test for cross-species immunity.

An alternative vaccine approach is to define the conserved epitopes in related antigens from different species and focus the immune response on these epitopes. This hinges on the hypothesis that despite the extreme antigenic diversity in *Plasmodium*, there exist evolutionarily conserved epitopes that can elicit protective antibodies or stimulate cross-reactive T-cells. Richie argued that selection pressure would favor antigenic diversity in species that infect the same host to avoid cross-species immunity that could eliminate both species (7). The continued scourge of malaria globally supports this tenet.

It is clear from the analysis of *Plasmodium* genomes that the immunodominant antigens in all species are highly polymorphic. Yet if we consider the functions of these diverse antigens, they are largely restricted to the pathogenesis of a particular species. For instance, the PfEMP1 virulence factors mediate sequestration of iRBCs to different tissues as a mechanism of immune evasion. Sequestration may have evolved to enhance the virulence of *P. falciparum* over other species that co-circulate in a given population and compete for the same host. But since the PfEMP1 family is unique to *P. falciparum* (and *P. reichenowi* in primates), the diversity among the members of the PfEMP1 family hinders the acquisition of strain-transcending immunity only within this species. This is also exemplified by the highly polymorphic proteins involved in erythrocyte invasion. *Plasmodium* species exhibit host cell tropism for different types of RBCs and evolved parasite ligands that bind to host receptors on those specific cells. In *P. vivax*, the PvDBP ligand interacts with the Duffy antigen receptor for chemokines (DARC) to invade reticulocytes; there is extensive diversity in the PvDBP domain that interacts with DARC. These polymorphisms are selected to evade immune responses that would block invasion of reticulocytes, yet they remain specific to *P. vivax* and do not impact other species that require different ligand-receptor interactions for invasion.

Despite the selection for variation in the immunodominant antigens within each species, many of these proteins evolved from ancestral homologs. The PfEMP1 and PvDBP proteins share a common protein architecture which includes DBL domains. While the functions of proteins with DBL domains diverged significantly within and across species, these domains may nevertheless have conserved epitopes that are essentially 'evolutionary relics'. These epitopes are probably not highly immunogenic and would induce antibodies with lower avidity toward their heterologous counterparts. They may even be cryptic in some proteins, which could explain the non-reciprocal nature of cross-species immunity observed in so many of the human and animal studies (**Figure 2**). Based on the data reviewed here, we propose that these epitopes may be more exposed in less virulent parasites and cryptic in more virulent ones. This could provide a competitive advantage for the benign parasite and ensure survival of the host.

Strategies and Challenges

We propose that vaccination can refine and amplify a cross-species immune response to target heterologous antigens. There are a number of potential vaccine targets identified already that elicit cross-reactive antibodies (**Figure 1**) and certainly new targets to discover (**Figure 3**). The first step to identify new cross-reactive B-cell epitopes is to test sera for reactivity to heterologous parasites (e.g. by IFA). Cross-reactive sera should then be assessed for functional activity against the heterologous parasite. This could involve testing the sera in various *in vitro* assays to measure effects on invasion, sequestration, transmission-blocking activity, etc. Most of these assays measure antibody function, with only indirect assays to measure T-cell mediated responses (e.g. cytokine production) (85, 86). Even the more established antibody-based assays, such as the growth inhibition assay, vary in terms of validity and predictive value, and are largely antigen and strain-specific (87). Assays to measure adhesion-blocking activity also vary with the format; for example, the anti-adhesion activity of VAR2CSA antibodies varies significantly when compared using a static inhibition of binding assay, a flow-based assay, and a placental perfusion assay (88). Nevertheless, these assays can provide insight into the pathway blocked by those antibodies and generate hypotheses of which antigens are likely targets. Once the target protein is identified (through biochemical and/or immunological methods), antibodies specific to this antigen can be purified from the sera or generated as mAbs. These antibodies can be characterized in terms of their cross-reactivity (titers, avidity) and their functional activity against the heterologous parasite.

To translate these findings into vaccine candidates, the cross-reactive epitope can be mapped using a variety of approaches. B-cell epitopes may be linear but are more likely to be conformational if they represent structurally related epitopes. Conformational epitopes are certainly more challenging to map but advances in structural and computational biology provide valuable tools that support rational vaccine design [reviewed in (89)]. For example, cross-reactive human or mouse mAbs can be co-crystallized with the target protein to map the epitope empirically (75). These antibodies can also be used to screen peptide libraries (conformationally constrained or linear) or tested against mutant recombinant proteins to identify the epitope that mediates cross-reactivity. In parallel, computational approaches can be applied to protein databases to predict conserved epitopes. This technique was recently adopted to predict conserved linear and discontinuous epitopes in CSP and MSP-1 shared between the *P. falciparum* and *P. vivax* orthologs (56). Computational modeling can also guide the formulation of vaccines to enhance immunogenicity and to elicit broadly neutralizing antibodies. Computational simulations of affinity maturation applied to the antibody response to *P. falciparum* AMA-1 revealed that polyvalent vaccines promoted cross-reactive antibody responses to shared epitopes (across strains; AMA-1 from different species was not included) (90). In future, entire proteomes and epitope libraries spanning the evolutionary spectrum of Plasmodia can be probed using artificial intelligence and machine learning to discover targets of cross-species epitopes. It is important to note that the process of identifying and refining the epitope is iterative and each approach can complement and inform the other.

Once a cross-species epitope is mapped, the next step is to reproduce the epitope synthetically such that it can elicit

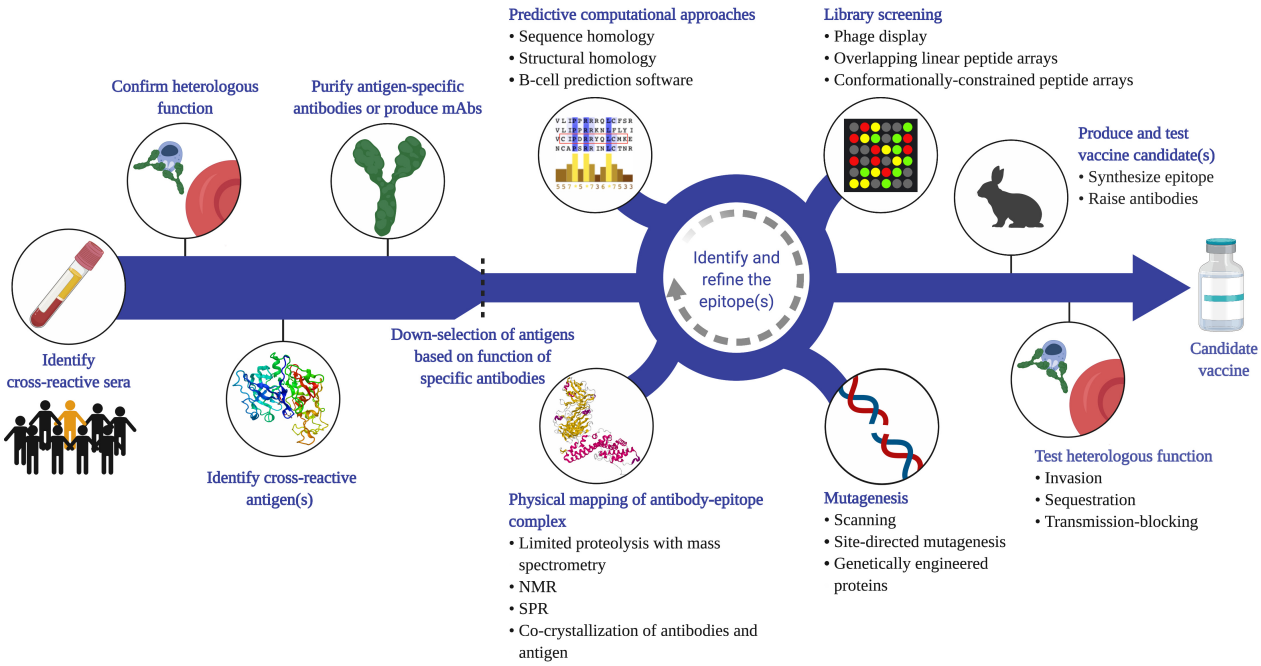

FIGURE 3 | Proposed strategy to identify cross-species vaccine candidates based on cross-reactive B-cell epitopes. Given that cross-species immunity is a rare event in naturally exposed populations, a large number of samples from endemic populations will need to be screened (e.g. by IFA or flow cytometry) to identify sera that recognize heterologous parasites. Antibody function against the heterologous species should then be confirmed (e.g. invasion, cytoadherence, transmission-blocking assays), and the antigen that mediates this functional cross-reactivity identified. This can be achieved through a variety of methods, including depletion or competition experiments. Antigen-specific antibodies can be affinity-purified from sera, or monoclonal antibodies generated using PBMCs from naturally exposed individuals or from animals vaccinated with the antigen. Functional analysis of these antibodies can then be used to down-select candidate antigens before applying a variety of empirical approaches to map the cross-reactive epitope. Phage and peptide libraries can be screened with the cross-reactive antibodies. Mutagenesis techniques, such as site-directed mutagenesis or alanine scanning of recombinant proteins can map residues that are critical for antibody binding. Physical mapping, such as co-crystallization of the antigen-antibody complex, is another powerful approach to map the contact residues within the epitope. These experimental tools can be integrated with computational analysis of the antigens from each species. Once a putative cross-reactive epitope is identified, the next step is to generate a recombinant protein or synthetic peptide that recapitulates this epitope, raise epitope-specific antibodies in animals, and test for cross-reactivity and function *in vitro*. It is important to note that the process of identifying and refining the epitope is iterative and each approach can complement and inform the other to yield potent, functional cross-species antibodies. Created with Biorender.com, including crystal structures PDB accession numbers 1SME and 6R2S.

functional antibodies or protective cellular responses against the heterologous epitopes in other species. This can be achieved with engineered recombinant proteins that expose the epitope preferentially (e.g. 91), linear epitopes conjugated to carrier peptides, and for conformational epitopes, this is feasible with the use of peptide scaffolds that restrict the conformation of peptides as immunogens (e.g. 92). An alternative delivery platform is the use of transgenic parasites from one species engineered to express antigens from a different species (93). The success of these approaches will depend on the fine specificity of the antibodies and their avidity for the heterologous epitopes. The avidity of cross-species antibodies observed in human and animal infections is generally low but different immunization strategies can be adopted to promote affinity-maturation, including the choice of adjuvant, delivery platform, dosing, and boosting schemes (2). As such, several rounds of identification, designing and testing may be required to produce potent, functional cross-species antibodies.

One outstanding question is whether these immune responses would be boosted by natural infection with heterologous species. This may depend on a number of variables including the intensity of parasite transmission, host genetics, and immune regulation. We hypothesize that if the epitope is truly cross-reactive, then memory B- or T-cells may be expanded by exposure to the heterologous epitope even if it is not immunogenic in that species. This phenomenon was recently reported for a cryptic epitope in group A streptococcus where immunization with the conserved peptide was boosted by natural infection with different bacterial strains (94). Similar vaccine strategies are being adopted against cryptic epitopes in Ebola antigens (95), and toward the development of a universal influenza vaccine (96–98).

CONCLUSION

The slow progress in developing a malaria vaccine underscores the many challenges with a traditional vaccine approach. We need to consider alternative, yet complementary strategies. Thus, exploiting rare immune mechanisms like cross-species immunity are worthy of consideration and with our current tools, this is more amenable than ever before. We don't expect this approach to yield a vaccine that provides sterile immunity

to malaria; but if we could emulate the reduction in disease severity observed with heterologous infections in humans and in animal studies, this vaccine could reduce mortality in the most vulnerable populations and allow natural, strain-transcending immunity to develop.

AUTHOR CONTRIBUTIONS

CM and SY jointly wrote the manuscript.

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The Impact of Type 1 Interferons on Alveolar Macrophage Tolerance and Implications for Host Susceptibility to Secondary Bacterial Pneumonia

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That macrophages adapt to environmental cues is well-established. This adaptation has had several reiterations, first with innate imprinting and then with various combinations of trained, tolerant, paralyzed, or primed. Whatever the nomenclature, it represents a macrophage that is required to perform very different functions. First, alveolar macrophages are one of the sentinel cells that flag up damage and release mediators that attract other immune cells. Next, they mature to support T cell priming and survival. Finally they are critical in clearing inflammatory immune cells by phagocytosis and extracellular matrix turnover components by efferocytosis. At each functional stage they alter intrinsic components to guide their activity. Training therefore is akin to changing function. In this mini-review we focus on the lung and the specific role of type I interferons in altering macrophage activity. The proposed mechanisms of type I IFNs on lung-resident alveolar macrophages and their effect on host susceptibility to bacterial infection following influenza virus infection.

Keywords: type I IFN, trained immunity, alveolar macrophage, lung viral infection, secondary bacterial pneumonia, epigenome, tolerance

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INTRODUCTION

Bacteria entering the respiratory tract are generally tolerated well in healthy adults and their growth contained by the host commensal microbiome, antimicrobial peptides, phagocytic cells (predominantly macrophages), mucus entrapment, and ciliary clearance. Some bacteria associated with respiratory tract infections are part of the normal microbiome in health, such as *Streptococcus pneumoniae*, *Haemophilus influenza,* and *S. aureus* (1–3). However, severe consequences arise when the lung microenvironment is perturbed in some way. Perturbations can include underlying congenital abnormalities (e.g., primary ciliary dyskinesia), underlying chronic disease (e.g., asthma, chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis), the effect of the aging process, the premature lung and previous severe infections (4). In all cases, the outcome depends on the severity of the perturbation, the rate of bacterial growth, and whether the bacterium is contained in the airspaces or invades the lung tissue and systemic circulation.

Containment of bacteria relies on effective physical and chemical barriers, but also a timely immune response. Any delay in immunity allows the growth of bacteria to an over-whelming level. It is interesting to note that conditions associated with bacterial out-growth occur in situations where the lung has a heavy infiltration of the very cells (macrophages and neutrophils) required to clear the micro-organism, which suggests they are not functioning properly (5). The function

and phenotype of any immune cell is influenced by the local microenvironment and the needs of the tissue at that time. We referred to this adaptation as "innate imprinting" in 2004 (6) that was superseded by the term "trained immunity" (7–10). However, the terminology continues to evolve and now trained immunity represents a "primed" state that is beneficial, whereas the more immune paralyzed state (as observed following viral infection of the lung) is referred to as a "tolerant" state. Trained/tolerant innate immunity is important in health, disease and disease resolution. The molecular mechanisms of trained immunity in health have been described extensively elsewhere (11). Here we will describe how alveolar macrophages are tolerised during and following inflammation with a specific emphasis on the role of type I interferons (type I IFNs).

DO MYELOID CELLS ADAPT?

Specificity and adaptation were once the hallmark of adaptive immunity alone. However, epidemiological studies as early as 1946 recognized that the *Mycobacterium tuberculosis* vaccine, BCG, also protected against childhood mortality caused by antigenically indistinct organisms, suggesting "adaptation" of cells of the innate, rather than adaptive, immune system (12, 13). Since then more recent studies have shown that innate immune cells can display adaptive characteristics (11). In terms of generating a specific response, it could be argued that pattern recognition receptors (PRRs), expressed by innate immune cells, confer specificity. PRRs are germline-encoded receptors and include the toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectins (14), among others. These receptors vary widely in the ligands that they bind to, allowing them to detect a substantial range of molecular patterns, known as pathogen- and damage-associated molecular pathogens (PAMPs and DAMPs, respectively) (15). This activates both divergent and convergent downstream signaling pathways enabling a tailored response to a specific pathogen (14). Furthermore, it is now recognized that innate immune cells, for example myeloid cells (7, 8, 16), NK cells (17, 18) and epithelial cells (19), can acquire "memory", characterized as a heightened and quicker response upon re-exposure to a pathogen. Innate immune memory is well-defined in organisms that lack an adaptive immune system, including plants and invertebrates (20, 21). This is more controversial in vertebrates, partly due to the relatively short half-life of innate cells, which in the case of monocytes can be up to 1 day in the circulation (22). However, the presence of innate immune memory in monocytes has been observed for up to 3 months (13) and for macrophages 6 months or more (23). This innate immune memory or trained immunity likely serves as an evolutionary survival advantage with the innate immune system primed to combat a secondary pathogen encounter (11). However, training can lead to deleterious consequences if the outcome is a macrophage that is tolerant to stimulation. A slower macrophage response likely protects the host from further tissue damage, prioritizes a reparative state and prevents the development of autoimmunity. In the case of severe influenza virus infection, upon resolution macrophages are unable to respond quickly enough to curtail bacterial load leading to complications of secondary pneumonia (24).

TYPE I IFNs

There are many mechanisms associated with susceptibility to bacterial complications following lung viral infection. However, type I interferons (IFNs) stand out as particularly important as they directly impair, or lead to downstream consequences affecting, bacterial clearance (**Figure 1**) (25–30). All three types of interferons (Types I – III) play a major role in innate and adaptive immunity (14). Of the eight (-α, -β, -δ, -ε, -ζ, -κ, -τ, and –ω) type I IFNs, the -α, -β forms, which bind to the IFNAR receptor complex (IFNAR1 and IFNAR2), have received the most attention with regards to lung viral infection (32). Receptor binding recruits janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) that leads to the phosphorylation of Signal Transducer and Activator of Transcription (STATs). Phosphorylation leads to homodimers and heterodimers; the precise combination dictating the final transcriptional outcome. STAT 1 and 2 heterodimers bind to IRF9 to form the ISG (Interferon Stimulated Gene) factor 3 complex−9 (33, 34). Type I IFNs also activate the p38-associated MAPK (mitogen-activated protein kinase pathway) (35). Type I IFNs have a myriad of functions in the lung where they are both crucial for the clearance of viral infection and resolution of inflammation. However, it is these diverse functions that are thought to contribute to host susceptibility to bacterial infections following viral infection.

TYPE I IFNs AND HOST SUSCEPTIBILITY TO SECONDARY BACTERIAL INFECTION

The contribution of type I IFNs to host susceptibly to bacterial infection is well-established (**Table 1**). In 2001, Biron's group defined a role for IFN α/β in viral-induced sensitization to bacterial products (36). Viral mimics, such as Poly I:C, also impair anti-bacterial immunity by induction of type I IFNs (37). Since then the field has expanded rapidly to show that type I IFNs decrease neutrophil chemoattractants (CXCL1/2) (25, 26), reduce IL-17 producing γδ T cells (27), and impair CCL2-mediated recruitment of macrophages following viral infection (28). Furthermore, depending on the context type I IFNs can promote or inhibit NLRP3 inflammasome activation, causing either an increase in IL-1β that limits γδ T cell activity with subsequent susceptibility to *S. pneumoniae* (27) or decreases IL-1β production enhancing susceptibility to *S. aureus* (29, 30), respectively. Additionally, type I IFN induced by viral infection alters cellular metabolism that may favor bacterial replication, uptake and adhesion (38). These mechanisms have been reviewed extensively elsewhere (39).

The immune suppressive outcome of enhanced type I IFNs is exemplified by strategies to inhibit its action. Inhibition of IFN receptor I- and III-associated TYK2 restores anti-bacterial immunity in a human *ex vivo* lung co-infection model (40). An absence of STAT2 that is downstream of IFN-αR 1/2 makes

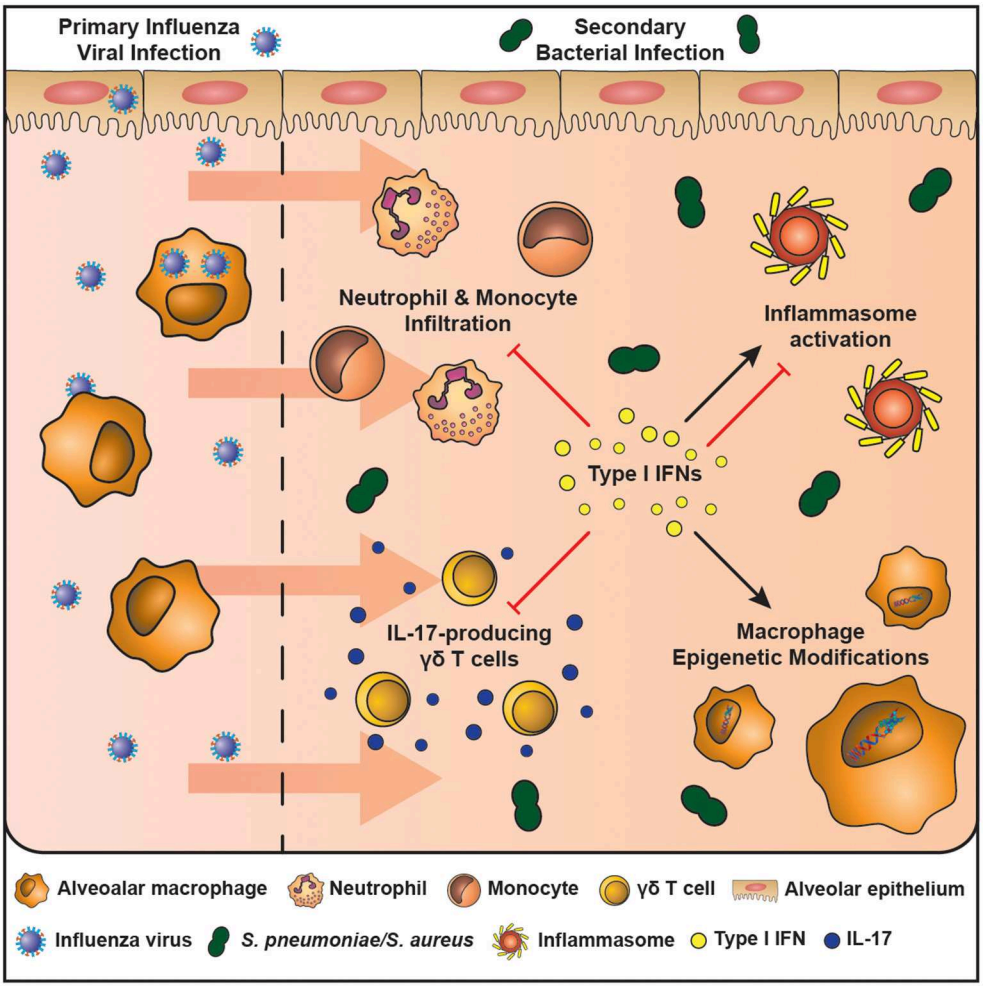

FIGURE 1 | The mechanisms of enhanced host susceptibility to secondary bacterial infection by type I IFNs. Airway epithelial cells and alveolar macrophages are cells of the innate immune system that are at the first line of defense against infection in the airspaces. The influenza virus activates pattern recognition receptors expressed by airway epithelial cells and macrophages and leads to the production of type I IFNs, which are crucial in combating the infection. However, type I IFNs also induce an immunosuppressive state in the resolution phase of infection that enhances host susceptibility to secondary bacterial infection. These mechanisms include: (1) inhibition of IL-17-producing γδ T cells, (2) Induces macrophage epigenetic modifications, (3) Induces or inhibits inflammasome activation in a context-dependent manner, (4) Inhibits neutrophil and monocyte infiltration. These mechanisms result in a lung environment ill equipped to fight an increasing bacterial burden.

influenza infection more severe, but prevents the development of secondary bacterial pneumonia (41). Furthermore, blocking Toll-like receptor 4 (TLR4) after influenza virus infection decreases bacterial growth by reducing IFNβ (26). Type I IFN induction may also contribute to the risk of bacterial infection following the administration of anesthetics prior to surgery. Infectious risk due to the immune modulatory effects of anesthetics delays surgical procedures in patients suspected of a respiratory infection. However, not all anesthetics cause this problem (42) and halothane actually reduces bacterial burden in influenza infected mice by decreasing type I IFN in the mouse lung (43). These observations suggest that type I IFN-induced tolerance following severe lung viral infection, although beneficial in limiting excess tissue damage and restoring tissue to homeostasis, results in a macrophage unable to deal with a growing bacterial burden.

TYPE I IFNs AND THE RESTORATION OF THE STEADY STATE

Type I IFNs are directly involved in important processes necessary to restore the lung to health. A reduction of inflammatory responses during apoptotic cell clearance is critical to prevent autoimmunity to self-antigens. Type I IFN receptor signaling induces suppressor of cytokine signaling (SOCS) 1 and 3 activation during efferocytosis of apoptotic cells by the receptor tyrosine kinase AXL (44). The combination of AXL and IFNAR1 signaling causes reduced macrophage responses and subsequent bacterial complications (45, 46). Furthermore, macrophages are also "tolerised" during the uptake of extracellular matrix turnover by-products; again an important function to restore homeostasis (47). The glycosaminoglycan, hyaluronan for example, is a

TABLE 1 | The effects of viral—induced type I IFN on the inflammatory response to secondary bacterial infections.

	Primary viral infection	Secondary bacterial infection	Type I IFN-mediated effects (↓ = decreased; ↑ = increased)	References
Inflammatory response	H1N1 influenza A/PR/8/34 virus (PR8)	Type 3 S. *pneumoniae*	↓ Neutrophil chemoattractants (CXCL1/2)	(25, 26)
	Influenza virus A/X31 (H3N2)	Type 3 S. *pneumoniae*	↓ IL-17-producing gamma delta T cells	(27)
	H1N1 influenza A/PR/8/34 virus (PR8)	Strain P1121, S. *pneumoniae*	↓ CCL2- mediated recruitment of macrophages following viral infection	(28)
	Influenza virus A/X31 (H3N2)	Type 3 S. *pneumoniae*	↑ NLRP3 inflammasome activation	(27)
	Influenza A/PR/8/34 H1N1	Methicillin-sensitive *S. aureus*	↓ NLRP3 inflammasome activation	(29, 30)
Epigenetic modifications	H1N1 influenza A/PR/8/34 virus (PR8)	Type 3 S. *pneumoniae*	↑ Production of the methyltransferase Setdb2 ↑ H3K9me3 chromatin marks at the CXCL1 promoter ↓ Neutrophil Infiltration	(31)

prevalent extracellular matrix component in the lung (48), but it suppresses alveolar macrophage activity and is maintained at a higher level following resolution of a severe viral infection (49). Similarly, versican, a chondroitin sulfate proteoglycan, is expressed at low levels in the healthy lungs, but upregulated by TLR agonists LPS and Poly I:C and requires TLR, TRIF and type I IFN signaling. In turn versican up-regulates IL-10 and IFNβ, leading to an immune suppressive state (50). Therefore, repairing the damaged lung and restoring the steady state, impairs inflammation and involves type I IFNs. This raises the possibility that trained immunity in macrophages simply represents a change in function from inflammation to homeostatic maintenance.

EPIGENETIC MODIFICATIONS IN TRAINED IMMUNITY

The longevity of alterations in lung immunity following severe viral infection is surprising considering the relatively short life of innate immune cells. However, alveolar macrophages in particular, turnover relatively slowly in health (51). Of particular relevance to the altered reactivity of alveolar macrophages, is their re-wiring by epigenetic changes (52). Epigenetic changes are mediated by (micro) miRNAs, DNA methylation, and histone modifications, amongst others and regulate chromatin accessibility (53). Chromatin accessibility determines which genes are visible and therefore impacts on cellular signaling and gene expression.

Monocyte/Macrophage adaptation is accompanied by fundamental epigenetic changes (54, 55) and is often associated with alterations in cellular metabolism (56, 57). Trained monocytes, producing excess TNFα and IL-6 protect RAG-/- mice (lacking functional T and B lymphocytes) against reinfection with *Candida albicans* due to stable histone trimethylation at H3K4 (8). Candida binding to Dectin-1 causes stable changes in histone trimethylation at H3K4 and increases the immune responsiveness of monocytes (8). Similarly, chromatin modifications by BCG vaccination provide protection to unrelated infections (13). Tolerance induction in macrophages cultured with LPS results in methylation at H3K9me2 and H3K9me3 and protects against S. *aureus* infection (58). Looking beyond pro-inflammatory processes, it is clear that in tolerised macrophages not all genes are repressed in all circumstances. For example, LPS-stimulation of murine macrophages *in vitro* represses pro-inflammatory genes, but enhances genes encoding anti-microbial effector proteins (16). However, this is often not the case *in vivo*, where reduced anti-bacterial immunity and macrophage effector function are observed following viral infection. This discrepancy, represents an opportunity since it suggests that some stimuli lead to a different macrophage outcome. A recent study of influenza infection followed by a S. *pnemoniae* strain lacking the major virulence factor pneumolysin, shows that not all macrophages are affected equally and that long term epigenetic changes differ between recruited and resident macrophages (59). Understanding how to achieve a bactericidal vs. an anti-inflammatory macrophage outcome could provide strategies to combat post-viral bacterial pneumonia.

TYPE I IFN-INDUCED EPIGENETIC MODIFICATIONS

Type I IFN modification of the epigenetic landscape is mostly via their regulation of interferon-stimulated genes (ISGs) (60, 61). ISGs encode a wide range of proteins that restrict viral infection and spread, including inhibition of viral transcription, translation and replication, the degradation of viral nucleic acids and the alteration of cellular lipid metabolism (62, 63). Approximately 2,000 human and mouse ISGs have been identified and cataloged in the Interferome database (64). All classes of IFNs have overlapping ISGs (65, 66) and so it remains unclear how ISGs are regulated in order to produce a unique and tailored response to a given pathogen. Epigenetic modifications are proposed as one mechanism by which ISG transcription can be context specific (65). The ISGs induced may depend on the cell type, the exposure of the cell to other stimuli, such as PAMPs or DAMPs, or the strength and duration of the interferon stimulus. All these variables may affect the chromatin landscape and provide another level of ISG regulation to different environmental cues. Evidence shows that enhanced transcription of ISGs upon re-stimulation is not due to increased expression of the required transcription factors or IFN signaling molecules, but rather as a result of altered chromatin marks at ISG promoters, thereby priming or repressing certain ISGs. Of the 1,000 s of ISGs known,

only half are reported to become primed or display "memory" upon restimulation (61). Other inflammatory factors present in the microenvironment also affect the profile of ISGs available. For example, in response to LPS, type I IFNs prevent the silencing of inflammatory genes driven by prior TNF exposure of macrophages. This is mediated by an altered chromatin state, with increased recruitment of H4ac and H3K4me3 histone marks that are generally associated with transcriptional activity, and increased chromatin accessibility at tolerised genes (60). In addition to driving alterations in the epigenome, type I IFNs can also be regulated by epigenetic modifications. For example, miR146a (67), and miR26a (68) promote type I IFNs and reduce influenza infection in experimental models, whereas miR29a reduces IFNAR1 and has the opposite effect (69).

MANIPULATION OF THE EPIGENOME TO REVERSE TOLERANCE IN MACROPHAGES

An important aspect of viral-induced macrophage tolerance to consider is whether it can be overcome or reversed in order to unleash the full inflammatory potential of macrophages and promote anti-bacterial responses. One possibility could be via manipulation of epigenetic changes. For example, histone modifications are reversible and therefore can be altered. The methyltransferase Setdb2 is an ISG that regulates the production of the neutrophil chemoattractant CXCL1. Deletion of Setdb2 decreases H3K9me3 chromatin marks, releases the CXCL1 promoter from inhibition, enhances airway neutrophil infiltration and reduces susceptibility to secondary *S. pneumoniae* (31). Furthermore, β-glucan can overcome the tolerised phenotype of macrophages following LPS exposure (70) and monocytes from experimental endotoxemia in healthy volunteers. This suggests that it is possible to improve the antibacterial function of macrophages. A tolerance phenotype is also observed in other cells. Airway epithelial cells, for example, are also refractory to TLR agonists following stimulation that can be restored by histone deacetylase inhibitors (71). Although not specifically identified to our knowledge, it would be interesting to determine whether these inhibitors could potentially reverse macrophage tolerance and reduce susceptibility to secondary bacterial infections.

TYPE I IFN TREATMENT AND THE PREVENTION OF BACTERIAL SUPER-INFECTIONS

The post-viral lung effects of type I IFNs span multiple bacterial species, including *Streptococcus pneumoniae* (25), *Pseudomonas aeruginosa* (72), *Staphylococcus aureus* (73) [including multi-drug resistant forms (41)] and *Escherichia coli* (74). Furthermore, the preceding viral infection need not be in the lung. For example, systemic Lymphocytic choriomeningitis Virus (LCMV) infection causes apoptosis of granulocytes in the bone marrow leading to reduced recruitment of neutrophils to the airways during Listeria *monocytogenes* or *S. aureus* infection (75). Therefore, manipulation of type I IFNs may represent a therapeutic option once bacterial complications arise following severe viral lung infection. Targeting of type I IFN responses is currently used in the treatment of several inflammatory and autoimmune diseases. For instance, IFNβ is an effective therapy for multiple sclerosis patients and IFNα has been approved for the treatment of hepatitis B and C (76). In contrast, the blockade of the type I IFN receptor with anti-IFNAR, has been an attractive therapeutic for autoimmune diseases including systemic lupus erythematosus (SLE) and rheumatoid arthritis, as these diseases are characterized by a profound IFN gene signature (77, 78). However, difficulties in developing effective therapies that target the type I IFN system relies upon selecting the specific type I IFN to administer or block, and the timing of drug delivery, which can lead to opposing outcomes. This is observed by the pro-inflammatory and immunosuppressive mechanisms that type I IFNs can generate in the tumor microenvironment. Although IFNα immunotherapy has proven effective in the treatment of hematological malignancies (79, 80), type I IFN treatment of solid tumors has shown less potential (81). Conversely, type I IFN inhibition can promote an anti-tumor responses by unleashing the inflammatory potential of exhausted T cells and removing the requirement for combinatorial immune checkpoint inhibitor immunotherapies (82). Further understanding of the roles of individual interferons in different inflammatory contexts and the divergent downstream signaling pathways they trigger is still required to generate effective treatment options. Currently, research is lacking for targeting type I IFNs to treat secondary bacterial pneumonia. However, studies suggest that targeting the epigenome of ISGs may be a more successful avenue of investigation. This would more likely limit potential negative side effects that may arise from removing type I IFNs themselves.

CONCLUSION

Type I IFNs clearly play a central role in bacterial super infections following lung damage, particularly that caused by pulmonary viral infection. Here we have focused on the effect of, predominantly, influenza infection on macrophages. However, similar processes may exist following infection with other respiratory viruses, such as respiratory syncytial virus. Collectively, the evidence suggests that overcoming type I IFN driven immune suppression may be beneficial for viral-induced bacterial super infection. Anti-IFNAR (e.g., Sifalimumab) is already used in the treatment of SLE (83) and could be repurposed for post-viral lung conditions. However, any strategy would need to be carefully timed and type I IFN administration during influenza infection may enhance viral immunopathogenesis. Bacterial infections mostly arise when the bulk of viral titer has been eliminated. Sometimes there is a sufficient and visible window between viral infection and bacterial outgrowth that would allow timed treatment to be administered. However, ultimately the problem is dependent in the first place on the severity of the viral infection. Studies to date show that any strategy that reduces the impact of lung viral infection reduces the chances of developing subsequent bacterial complications. Vaccination would therefore still seem the best policy; as long as any attenuated forms do not induce excess type I IFNs themselves.

Finally, we should remember that macrophages attune to the needs of the tissue. Their trained/tolerant/primed state is therefore not abnormal, but rather represents a macrophage that has to first inflame to recruit immune cells, then change to professionally instruct them and finally clear up the mess afterwards.

AUTHOR CONTRIBUTIONS

EC and TH drafted the manuscript, made substantial contributions to the conception and design of the work, approved the submitted version of the manuscript, and agreed to be accountable for all aspects of the work.

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Virus-Induced T Cell-Mediated Heterologous Immunity and Vaccine Development

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Heterologous immunity (H.I.) is a consequence of an encounter with a specific antigen, which can alter the subsequent immune response to a different antigen. This can happen at the innate immune system level—often called trained immunity or innate immune memory—and/or at the adaptive immune system level involving T memory cells and antibodies. Viruses may also induce T cell-mediated H.I., which can confer protection or drive immunopathology against other virus subtypes, related or unrelated viruses, other pathogens, auto- or allo-antigens. It is important to understand the underlying mechanisms for the development of antiviral "universal" vaccines and broader T cell responses rather than just subtype-specific antibody responses as in the case of influenza. Furthermore, knowledge about determinants of vaccine-mediated H.I. may inform public health policies and provide suggestions for repurposing existing vaccines. Here, we introduce H.I. and provide an overview of evidence on virus- and antiviral vaccine-induced T cell-mediated cross-reactive responses. We also discuss the factors influencing final clinical outcome of virus-mediated H.I. as well as non-specific beneficial effects of live attenuated antiviral vaccines such as measles and vaccinia. Available epidemiological and mechanistic data have implications both for the development of new vaccines and for personalized vaccinology, which are presented. Finally, we formulate future research priorities and opportunities.

Keywords: cross-protection, immune memory, molecular mimicry, TCR repertoire, T cell epitope, virus-induced immunity, immunopathology, immunomodulation

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INTRODUCTION

Heterologous immunity (H.I.) arises from previous infections, which alter the immune response to a subsequent infection with a different pathogen (1). This mechanism is more likely to occur between closely related antigens, but may also occur among unrelated antigens, including bacteria, viruses, protozoa, and parasites. H.I. may alter the outcome of infections by providing sufficient immune protection or, in other cases, aggravating immunopathology (2).

H.I. is mediated by T memory cells or antibodies (**Figure 1**). Immunoglobulins recognize antigens when antigenic epitopes attach to paratopes (**Table 1**) at the antigen-binding site. Antibodies are potentially polyspecific, capable of binding different epitopes to various antigens. Furthermore, epitopes sharing similar sequences may bind to the same paratope, providing cross-protection (4). In the context of molecular mimicry, antibodies may also react to self-antigens, eliciting autoreactive immunopathology (5).

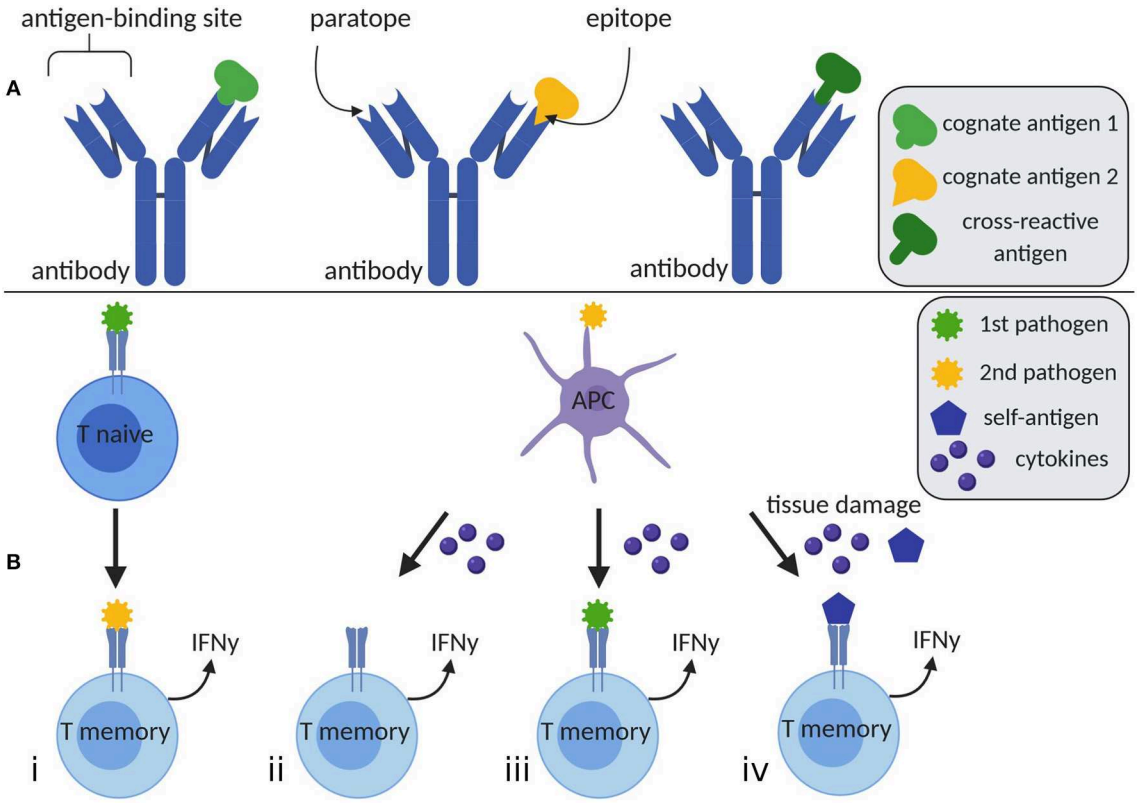

FIGURE 1 | Humoral and cellular mediated heterologous immunity. **(A)** A single antibody has the ability to bind distinct antigens 1 and 2 by different paratopes at the antigen-binding site. Furthermore, it is able to detect a cross-reactive antigen, whose epitope is similar to the one of antigen 1. **(B)** (i) T memory cells may be activated by an unrelated second pathogen, which is cross-reactive with the first encountered pathogen. (ii) The appearance of a second pathogen may elevate cytokine levels, which potentially lead to TCR-independent T cell activation. (iii) Simultaneous presence of cytokines and remaining antigens of previously encountered pathogens may stimulate T cells. (iv) High levels of cytokines and tissue damage due to inflammation or chronic diseases result in increased concentrations of self-antigens, which may be engaged by T cells. Created with BioRender.com, adapted from Welsh et al. (3). APC, antigen presenting cell; IFNγ, interferon γ.

Likewise, cellular-mediated H.I. plays a role in immunomodulation. This may be elicited via T cell receptor (TCR) cross-reactivity (one possible mechanism of H.I.), recognizing similar but distinct antigens or even autoantigens. T cells may also be activated non-specifically by cytokines [reviewed in (3)]. Cross-reactive antigens elicit an expansion of T memory cells, leading to a modified T cell memory pool, a change in patterns of immunodominance, and an altered hierarchy of T cell responses (6). This process heavily depends on the individual private specificities of TCR repertoires and ultimately results in a modified T cell response (7).

Trained immunity, also known as innate immune memory, is a recently described adaptation of innate immune cells following antigenic exposure. Epigenetic reprogramming leads to production of inflammatory mediators and a shift in cellular metabolism, providing an enhanced response to secondary stimulation [reviewed in (8)]. Thus, physiological processes such as mucosal tolerance, restriction of tissue damage, innate immunity maturation, and non-specific vaccine-mediated protection are achieved. Nevertheless, trained immunity can become maladaptive, causing immune paralysis or hyperinflammation [reviewed in (9)].

This review presents recent scientific findings regarding virus- or antiviral vaccine-induced T cell-mediated H.I. and thus provides some background for the discussion on benefits and risks of H.I. Implications for future research priorities for vaccine development are also considered.

VIRUS- AND ANTIVIRAL VACCINE-INDUCED T CELL-MEDIATED HETEROLOGOUS IMMUNITY

Influenza Virus

Naïve T cells of donors who self-reported as having no influenza A Virus (IAV) H1N1/09 exposure or influenza symptoms can recognize unique strain-specific epitopes using tetramer staining, whereas the same donors' memory T cells recognize conserved epitopes of the surface protein hemagglutinin (HA) (10). In H1N1/09 infected or vaccinated donors, the frequency of naïve T cells recognizing unique epitopes was significantly higher compared to conserved epitope-specific T cells (10). This has also been shown for $CD4^+$ (10) and $CD8^+$ T cells in mice (11).

Such observations suggest that H.I. influences the severity of infection (10). An age-related dampening of T-cell mediated

TABLE 1 | Glossary.

Heterosubtypic	Referring to different serotypes of influenza A virus, which are defined based on the surface proteins hemagglutinin (HA) and neuraminidase (NA).
HLA molecule	The human leucocyte antigen is located on cell surfaces and may present antigenic peptides to T cells.
Immunodominance	Only a few (immunodominant) epitopes are preferentially targeted by the immune response. The remaining epitopes evoke barely detectable T cell responses.
Molecular mimicry	An alignment of pathogenic structures with those of the host, which leads to immune evasion. However, structure similarity of pathogens and self-antigens may elicit autoreactive immune responses.
Paratope	A segment of an antibody's antigen-binding site, which complementarily binds an epitope.
Private specificity of TCR repertoire	TCR repertoires, which are different among individuals.
TCR repertoire	All T cell receptor clonotypes expressed by an organism.

H.I. was observed following a second heterologous infection in ferrets, which allowed the development of significant morbidity (12). These findings are in agreement with other studies focusing on aged animals, which showed that the clinical severity of primary infection is only moderately accentuated (13–16), while heterologous secondary infection induced severe disease (12, 17, 18). The induction of influenza virus-specific memory T cells is extensively investigated as they are responsible for heterologous protection in secondary natural infections with another influenza strain [reviewed in (19)]. Tissue resident memory T cells (T_{rm}) in the lung are particularly important in that respect as they are crucial for achieving optimal protection [reviewed in (19)]. Previous animal studies showed that a single intranasal live attenuated IAV vaccine application can evoke long-lasting protection to heterosubtypic challenge via T_{rm} response in the lung with a similar phenotype to those of infected mice (20). Several *in silico* approaches are available to identify T cell immunogenic regions on virus proteins. It has been demonstrated that epitope-rich regions within the nucleoprotein (NP) of the influenza virus contain highly conserved epitopes and therefore present promising targets for a T cell-mediated vaccine due to cross-reactivity with distinct strains (21). Gutiérrez et al. developed a computational method to compare the efficacy of conserved T cell epitopes (EpiCC), which may complement current methods for selecting the best composition of an associated vaccine (22). Furthermore, $CD8^+$ T cells recognizing different NP variants were associated with cross-reactive TCR clonotypes against distinct strains (23). This was shown for the immunodominant and abundant human epitopes $NP_{338-346}$ and NP_{44-52} (23). A structural analysis of the associated HLA molecules revealed adoption of similar conformation as a basis for cross-recognition (23).

Spleen cells from IAV-infected animals showed enhanced IFNγ production after *ex vivo* stimulation with the hepatitis C virus (HCV) derived peptide $NS3_{1073}$ (24). Such findings suggest a private repertoire of pre-existing memory T cells, which are reactivated after HCV infection (25). Cross-reactivity was also demonstrated in human peripheral blood mononuclear cells (PBMCs) of HCV positive patients with severe disease which responded to the IAV-specific peptide $NA_{231-239}$ (25). Additionally, PBMCs of hepatitis B virus patients were incubated with Epstein-Barr virus EBV-$BMLF1_{280-288}$ and IAV-$M1_{58-66}$ labeled tetramers and subsequently stained for TCR clones (26). The TCR repertoire of cross-reactive T cells recognizing IAV and EBV epitopes was broader compared to non-cross-reactive T cells and varied among individuals, further supporting an underlying private specificity (26). The concept of H.I. has recently been expanded to include allergens, following demonstration of IAV-mediated protection against allergen-induced experimental asthma (mediated by memory T cells) in a murine model (27).

Flaviviruses

The high degree of genetic sequence similarity among flaviviruses is known either to have a protective effect or to dampen the elicited secondary immune response [reviewed in (28)]. For Dengue virus (DENV), it is well-known that an infection with one serotype induces strong and long-lasting protective immunity against that specific serotype, whereas a second infection with a heterotypic virus commonly results in severe disease [reviewed in (29)]. Sub-neutralizing antibody concentrations from the first infection facilitate virus entry by promoting Fcγ-receptor uptake, resulting in antibody-dependent enhancement (ADE) of the infection. However, there is increasing evidence of a cross-protective cellular immune response between DENV and Zika virus (ZIKV) [reviewed in (29)]. Memory T cells isolated from DENV seropositive patients recognize both DENV- and ZIKV-associated peptides (30). Furthermore, DENV positive patients responded more strongly to a ZIKV infection compared to DENV negative subjects when assessed using T cell stimulation assays (30, 31). Mouse experiments have also shown, that DENV-exposed pregnant animals were protected against subsequent maternal and fetal ZIKV infection (32). This protection was conferred by $CD8^+$ T cells, limiting trans-placental transmission of ZIKV (32). Although cross-reactivity between DENV and ZIKV is the most prominent example, other flaviviruses, such as yellow fever virus (YFV) and Japanese encephalitis virus, also prime T cell responses toward a subsequent heterologous DENV infection in mice (33). In this context, the investigators identified homologous sequences between the flavivirus polyproteins. Peptides derived from the aforementioned sequences were used to prime antigen presenting cells, which were subsequently used to stimulate splenocytes of DENV immunized mice. Some of these peptides induced enrichment of T memory cells as well as IFNγ production and proliferation, confirming cross-reactivity (33).

Human Immunodeficiency Virus

Human immunodeficiency virus 1 (HIV-1)-specific $CD8^+$ T cell clones showed cross-reactivity against some of the other investigated HIV-1 epitopes (34). Additionally, three HIV-1-specific T cell clones recognized the A*02 restricted IAV matrix epitope GILGFVFTL (34). Furthermore, a sequence similarity between the known HIV-1 epitope HIV-Gag [SLYNTVATL [HIV-SL9]] and the HCV epitope HCV-NS5b [ALYDVVSKL

[HCV-AL9]] has been observed. HIV-SL9 specific T cells of HIV-1 patients, who were not co-infected with HCV, recognized the aforementioned HCV epitope and responded with IFNγ production and expansion (35).

Hepatitis C Virus

Cross-genotype protective immunity against HCV was first described in 2003 by Lanford et al. who showed that chimpanzees, which recovered from a genotype 1 infection, were subsequently protected from infection with other genotypes (including genotype 4 and combinations of genotypes 1–4). These genotypes express proteins of up to 30% amino acid variance (36). This finding, however, has been challenged by other investigators who showed that chimpanzees developed chronic disease after being re-challenged with other genotypes (37).

$CD8^+$ T cell cross-reactivity to NS3 epitopes of two different genotypes (1 and 3) was observed in a study with 53 anti-HCV positive injection drug users. Interestingly, $CD8^+$ T cells recognizing both genotypes were more frequent among HCV RNA negative patients than in those with detectable viremia, implying that $CD8^+$ T cell-mediated cross-reactivity may protect against chronic infection (38).

In another study, an HLA-restricted epitope (HCV NS3-1406) and its naturally occurring variants from different genotypes showed that the frequency of cross-reactivity between variants as well as their T cell priming capacities varied, depending on the genotype pair (39). Fytili et al. performed a similar study for another dominant HLA-dependent HCV $CD8^+$ T cell epitope (HCV NS3-1073), which was associated with clearance of acute infection, and detected cross-reactivity between the genotype 1 variant and variants of genotypes 4, 5, and 6 but not 2 and 3 (40). The level of cross-reactivity observed in this study could be predicted through *in silico* analyses of peptide-MHC complexes and TCR-interacting surfaces based on topology and electrostatic features (41).

The same dominant T cell epitope (HCV NS3-1073) was also found to induce immune response in approximately a third of >100 seronegative individuals upon *ex vivo* stimulation. The presence of $CD8^+$ T cells specific for that epitope was attributed to cross-reactivity with epitopes derived from other pathogens. These cells not only reacted to different genotype variants of that epitope but also to epitopes with little sequence similarity of other, unrelated viruses (cytomegalovirus, IAV, EBV) (42). Immunization with a recombinant adenovirus vector containing mycobacteria, Ebola and HIV antigens also led to T cell responses against HCV alongside the transgenic antigens (43). Cross-reactivity between an HCV and a human herpes virus peptide has also previously been demonstrated (44).

Other Viruses

Severe hand, foot and mouth disease is caused among others by enterovirus 71. A dominant capsid T cell epitope, which is highly conserved among enteroviruses, was identified and found to yield a cross-reactive, HLA-DR restricted response of human $CD4^+$ T cells to the poliovirus variant of this epitope (45). Human RV-specific $CD4^+$ T cells were shown to recognize epitopes shared among different RV strains (46). Human circulating RV-specific $CD4^+$ T cells recognized conserved RV capsid protein epitopes, and T cell-mediated cross-reactivity between different strains was demonstrated (47). Zhao et al. showed that airway $CD4^+$ T memory cells specific for a dominant, conserved epitope (SARS-N353) protect against both SARS- and MERS-CoVs and also against bat CoV in HLA transgenic murine models (48). Hepatitis E virus (HEV)-specific $CD4^+$ and $CD8^+$ T cell responses against different peptide pools from HEV1 were detected in acute HEV3 patients. A similar response against HEV3- and HEV1-peptide pools was detected in one subjectwith HEV1 infection (49). Finally, H.I. between the arenaviruses lymphocytic choriomeningitis virus and Pichinde virus was demonstrated in murine models and found to be T cell epitope and MHC class dependent (50).

DISCUSSION

Protection vs. Immunopathology

Overall, virus-induced H.I. appears to be an important determinant for the final outcome of infections and of a plethora of dysregulated immune responses such as in autoimmunity and allograft rejection. In this context, prior antigenic exposures may boost protective responses [e.g., (27)] or induce immunopathology depending on the balance between antigen load and efficiency of effector T cells, which in turn is influenced by a number of factors. For example, in the case of flaviviruses, it has recently become evident that distinct T cell populations, virus serotypes, sequence, and number of infections, and HLA background all shape the immunodominance pattern (29). Additionally, patterns of T cell cytokine response among patients with a secondary DENV infection were associated with severe (51, 52) or mild dengue (53, 54). Although heterotypic antigens were addressed only in one of these studies (52), such observations may indicate involvement of cross-reactive T cells in the clinical manifestation of DENV infections.

In addition to natural viral infections, antiviral vaccines may also drive T cell-mediated H.I. and have a major impact not only against the vaccine antigens but also on completely unrelated pathogens or other antigens. To date, epidemiological evidence supporting the role of live attenuated vaccines in T cell-mediated H.I. is associated with the measles (55–62), the vaccinia (63–66), and the oral polio vaccine (67–69). These vaccines reduced overall mortality and/or risk for asthma, malignancies, and unrelated infections. Furthermore, they induced changes in the numbers or proportions of T and B cells, which, depending on persistence of effects, may influence differentiation, proliferation or survival of associated cells. Non-specific effects of vaccines have often been found to be sex-specific and influenced by revaccination as well as maternal priming. In this regard, knowledge on the potential of specific T cell epitopes (for any given HLA background) to offer protection or cause pathology is crucial for vaccine design including elimination or inclusion of such peptides.

Implications for Vaccine Development

The ability to predict the magnitude and mechanism of T cell-mediated H.I. (**Figure 1B**) is crucial for specific vaccine design but also for decisions on public health and vaccination policies. Structural similarity between T cell epitopes seems to be important for eliciting cross-reactive responses. Nevertheless, seemingly distinct epitopes may also bind to the same TCR and induce H.I. This may be explained by the fact that sequence similarity is also dependent on the presence of biochemically similar amino acid substitutions (70). In the context of developing broadly cross-reactive vaccines against viruses with great antigenic heterogeneity, regions of highly conserved proteins among serotypes may elicit cross-reactive T memory cell responses. This approach along with large scale systematic monitoring of circulating strains, as in the case of influenza (in order to minimize mismatch with vaccine-contained strains) may increase vaccine effectiveness.

Besides their specific effect, it is now known that vaccines may also exert a non-specific influence on the immune system (71). For the diphtheria-tetanus-pertussis and measles vaccines, it was shown that the order of vaccination has an impact on overall morbidity and mortality (72). The concept that the most recently administered vaccine leaves a non-specific immunological imprint until subsequent immunization may guide changes in the recommended order of childhood vaccinations. Such changes could result in beneficial non-specific effects with minor changes of existing national vaccination schemes. Similarly, age at the time of (initial or booster) immunization with each existing vaccine may need to be reconsidered based on the accumulating knowledge on immunosenescence and effects of age on virus-induced H.I. Accordingly, time of vaccination has been linked to differences in T cell populations and strength and type of heterologous immune response (73, 74). Sex-specific differences in terms of protective non-specific effects of vaccines such as measles and vaccinia (64, 75–80) have also been described. Modification of vaccine composition (e.g., enrichment of particular proteins or epitopes) or conditions of administration (e.g., age, dose, number of immunizations) could potentially help us achieve the beneficial heterologous effects of vaccines without compromising their primary protective effects (vaccine specific). Indeed, adequate application of knowledge regarding vaccine-mediated H.I. brings us a step closer to precision medicine and personalized vaccinology. Administration of live attenuated vaccines to women as part of preconception health counseling is another measure, which could enhance protection of offspring in the first months of life.

The potential of virus- and antiviral vaccine-induced immunomodulation may also be exploited for novel applications such as preventing infections among elderly and immunocompromised populations or non-infectious inflammatory diseases. In this respect, the choice of a particular adjuvant or pharmacological modulator is also important since these may polarize T cell immune responses toward a specific cytokine output depending on the desired outcome, e.g., induction of T1 type of response for prevention of infection as well as allergies.

Future Research Priorities

The need for new vaccines with higher efficacy and broader and longer-lasting protection is driven by the moderate protection provided by current seasonal influenza vaccines against the included strains, zoonotic and pandemic influenza threats, and the challenge of complying with annual vaccinations. Several approaches are currently being investigated with varying results and distance from truly universal vaccines. The use of adjuvants, addition of neuraminidase, and inclusion of specific strains induce broader reactive immune responses albeit within the same virus subtype. Additionally, immunogenic influenza HA-stem constructs induce B cells which produce cross-protective antibodies, at least within a group of viruses. A particular promising approach for the development of truly universal influenza vaccines seems to be the induction of T cells reactive to internal viral proteins, primarily of T_{rm} in the respiratory mucosa for timely control of viral replication. Such approaches could also prove useful for developing vaccines against other respiratory viruses such as rhinoviruses. Similarly, knowledge gained from current studies of T cell responses against DENV/ZIKV infections at several time points, and with different clinical presentations and history of infection may inform strategies for developing pan-flavivirus vaccines. Indeed, there is already evidence for cross-reactive immunogenic epitopes contained in these viruses.

Properties of virus-induced H.I. may be leveraged beyond infection protection. We have previously shown an influenza virus-mediated protection over development of experimental asthma in a murine model. The protection was conferred by $CD4^+$ and $CD8^+$ T memory cells, which were transferred from animals previously infected with influenza or immunized with cross-reactive influenza peptides to sensitized mice before challenge with an allergen. Given the global prevalence of allergies, peptide immunization strategies early in life could potentially induce protective cellular immune responses against viruses and allergen-induced asthma, and complement existing vaccination schedules. Importantly, directing non-specific beneficial effects of existing live attenuated viral vaccines against other inflammatory disorders including cardiovascular disease and cancer could be a quantum leap in the fight against non-communicable diseases (65, 81–84).

Further immunological and clinical studies are needed to decipher vaccine-induced H.I.-mediated mechanisms and impact on morbidity and mortality contributing to health promotion. Associated potentiators such as booster vaccinations and maternal priming need to be examined carefully in different socioeconomic settings and with a sex-differential analysis (85).

AUTHOR CONTRIBUTIONS

CS and PN planned, structured, and edited the manuscript. PN searched the literature and integrated all contributions. All authors wrote distinct parts of the manuscript and critically read, reviewed, and approved the final version of the manuscript.

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