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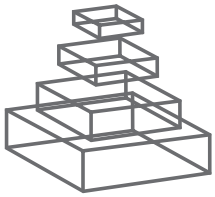

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GRANULOMA IMMUNOLOGY, PATHOLOGY

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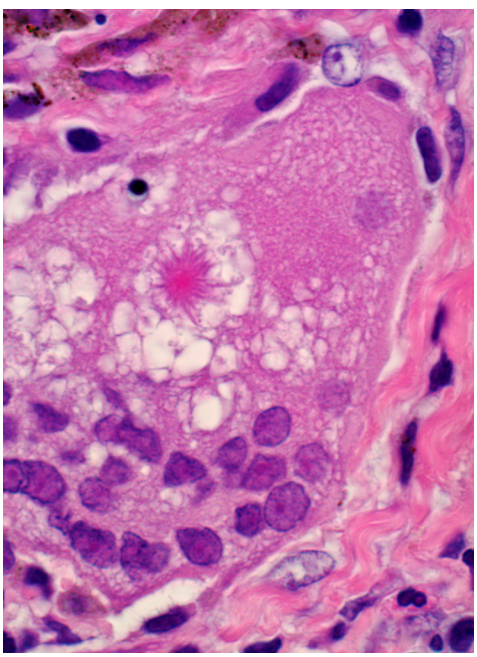

Figure reproduced from Chensue SW (2013) Chemokines in innate and adaptive granuloma formation. *Front. Immunol.* 4:43. doi: 10.3389/fimmu.2013.00043

The prototype of the granulomatous inflammation,-the mycobacterial tubercle,was first described by pathologists over 150 years ago.Granulomas are focal, chronic tissue inflammations in reaction to persistent microbial invaders,as well as chemical irritants.Immune granulomas are generated by CD4+T effector/helper cells. This Research Topic is initiated to provide a specific
state-of-the-art forum dedicated only to the various aspects of the granulomatous response.This includes the molecular basis of the initial interaction of the innate and adaptive immune responses:TLR,NRL and other innate receptors,the assembly and regulation of inflammasomes and inflammatory cytokine production.The fate of the mature granuloma is decided by the intensity and duration of the antigenic stimuli,and the action of the Th1,Th2 and Treg lymphocytes recruited to the site. The complexity of the ever expanding cytokine/chemokine web that sustains or regulates the inflammation and mediates the antimicrobial effect poses a major challenge for researchers to unravel and to elucidate.No less a challenge are the cytokine-activated macrophages, that play a major role in the elimination of the persistent pathogen.While doing this,macrophages release reactive oxygen and nitrogen free-radicals that cause tissue damage. Thus,yet another important task is the separation of the protective from the destructive effect of the inflammatory response.Macrophages comprise the bulk of the granulomas. Recent research shows that they are a heterogeneous population and can be divided into

more or less two distinct subpopulations.Thus,analysis of the function of M1- or M2-type macrophages with antimicrobial action-or others that activate fibroblasts for collagen deposition or conversely,control extracellular matrix turnover and fibrous healing of the inflammation is of much current research interest.Sarcoidosis,Crohn's disease and primary biliary cirrhosis comprise a distinct group of granulomatous diseases where controversy about the etiologic agent(s) and host responses still exists.Whereas in sarcoidosis and Crohn's disease microbial triggers have been proposed,in primary biliary cirrhosis the granulomagenic agent is still unknown.The progressive,often sever pathology affects single(intestines,liver) organs,thus little or no protective role can be assigned to the chronic granulomatous inflammations.In genetically predisposed individuals chronicity is believed to be sustained by the innate,adaptive,and autoimmune responses.Contributions to this Research Topic will promote knowledge on this complex protective vs/destructive response,and can lead to its improved handling in clinical practice.

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EDITORIAL
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New perspectives on ancient granulomas

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Keywords: pathogens, inflammation, granuloma, pathogen sequestration, pathogen protection, granuloma necrosis, pathogen dispersal

Granulomas (tubercles) were described by pathologists as the hallmark of tuberculosis (TB), an ancient, often lethal disease. The discovery by Koch of the bacilli [*Mycobacterium tuberculosis*, (M.tb)] as the causative agent of the disease initiated research that established the paradigm of the protective but also tissue-destructive nature of the granulomas. These Reviews provide an update on research in granulomatous infections/diseases and describe a shift into a new paradigm: the role of the pathogen in host-pathogen interaction.

The protective nature of the granuloma is based on phagocytosis and killing of the ingested pathogen driven by the innate and adaptive immune responses. On bacterial invasion the antigen presenting cells, macrophages via their pattern recognition receptors recognize the pathogen associated molecular patterns of the pathogen and initiate the defensive inflammatory response. Itoh et al. (1) present evidence that in the Bead-PPD lung granuloma model the innate TLR9 and the Dll4 Notch systems cooperate not only in the early recognition of M.tb patterns but also in skewing the incipient adaptive immune response toward the Th17 inflammatory cell activity. Granuloma formation is reliant on chemokines, the low MW proteins that recruit leukocytes to the focus of irritation and activate them. In Stephen W. Chensue's (2) laboratory the artificial granuloma model was established by means of antigen coated beads injected into Th1 or Th2 response-primed mice. The resulting lung granulomas showed the TH1 or Th 2 type cellular response. Chemokine receptor analysis of such granulomas showed lesion age-dependent heterogeneity of the response. It is suggested that chemokine's could be targeted for therapeutic intervention.

Ehlers and Schaible (3) give an overview of the M.tb granuloma research and the host-pathogen interaction. The protective role in TB of the granuloma is asserted, but reflecting the recent view M.tb is described to exploit the lesion for its dispersal and propagation. At the chronic stage of the disease bacilli within the lesion are dormant and adapt to low oxygen, pH, and nutritional conditions. The pathogen-induced strong TH1 type inflammation causes pathology: caseation, cavitation that allow the escape of the bacilli to infect a new host. The authors make the intriguing suggestion to focus on suppression of the intensity of inflammation rather than devising new vaccination modes that maintain the inflammatory response. Shaler et al. (4) describe the stages of the M.tb granuloma development with the appearance within the lesions of the non-phagocytic macrophage derivatives: foam, epithelioid, and giant cells. They take the negative view of the lesions because they contribute to bacillary persistence and based on the newly emerging zebra fish embryo model contend that granulomas may be dispensable. Lang (5) discusses the relevance of Mincle a C type lectin on macrophages that is the receptor for bacterial cord factor – a toxic glycolipid that incorporated into adjuvant induces granulomas. Linkage with TLRs could not be demonstrated and so far experiments with Mincle-deficient mice showed discrepant results related to anti-bacterial resistance. Lugo-Villarino et al. (6) give an account on the importance of the M1 M2 polarization that occurs during M.tb infection. Under the early TH1 (IFNg) influence M1 macrophages become strongly bactericidal. This shifts to a late Th2 (IL4, 10) M2 response with Arginase 1 expression that inhibits nitric oxide production resulting in poor or weak bactericidal capacity and the appearance of foamy macrophages that allow intracellular bacillary growth. Guirado and Schlesinger (7) confirm the protective role of the Th1-induced M.tb granuloma with strong M1 mediated bactericidal kill. They rightly stress the importance of the microenvironment in and around the granulomas that cause a variety of pulmonary pathologies in a single person and they acknowledge the changing dynamics between host and pathogen during the long course of the infection. They enumerate the various *in vivo* TB models, the *in vitro* efforts and the emergent *in silico* approaches whereby the complex granuloma response is being simplified by computer simulation.

Moore et al. (8) introduce visceral leishmaniasis a chronic tropical disease caused by the protozoan *Leishmania donovani*. In humans or mice host resistance is linked to efficient granuloma development. The murine model has several different characteristics. After infection the parasites are ingested by liver Kupffer cells which together with invariant natural killer T cells recruit monocytes. Both infiltrated CD4+ and CD8+ T cells are needed for parasite clearance. By 4D intravital imaging free movement of intralesional T cells was observed. Parasite clearance proceeds with no residual pathology (necrosis, fibrosis). The innovative computer modeling is being employed to simulate the effector cell populations and predict regulator cytokine activity.

Lundy and Lukacs (9) review the helminth-induced granulomatous schistosomiasis *mansoni*, where antigens secreted by the parasite eggs induce perioval granulomas that protect the liver from toxic egg secretions. The initial Th1 type inflammation, with chronicity is modulated to a Th2 response that significantly reduces granuloma size but enhances liver fibrosis. Downmodulation is mediated by T regulatory and CD5+ B cells that induce apoptosis of CD4+ T cells. It is also systemic for unrelated immune systems which has implications for vaccinations, allergy, and autoimmunity. Hams et al. (10) discuss the host parasite relationship in murine schistosomiasis. The parasite needs perioval granuloma formation for the successful egg excretion from the host. Host granulomas shield the liver from toxic egg secretions. But the predominant Th2 response generates eosinophile rich granulomas and excessive fibrous healing

that cause portal hypertension and intestinal bleedings. CD8+ T cells, B cells, and M2 type macrophages play a role in granuloma regulation. Egg antigens are also active at the innate response level: they suppress TLR triggered TNFalpha production by dendritic cells but activate the NLRP3 inflammasome with IL1beta production. Proteomic analysis of egg antigens revealed over 1000 proteins indicating the daunting task of assigning roles to fractions in inflammation or pathology.

The compiled Reviews show significant advances in granuloma research both in new methodologies and the emergence of new paradigm(s) in host-pathogen interaction. In the various models several contributors rightly reaffirm the essential protective role of the immune granulomatous response. However, in TB the quandary of the strong T cell-mediated anti-bacterial inflammation vs. the tissue-destructive pathological sequelae still remains unsolved. Similarly, in schistosomiasis there is no intervention that could prevent or reverse the strong fibrosis that replaces the perioval granuloma.

It is instructive to compare the various experimental models with pulmonary sarcoidosis, a granulomatous disease of humans with no known etiologic agent(s). Broos et al. (in review) survey the current literature that proves the immune basis of the disease. Clinical observations described innate responses with cytokine/chemokine secretion, macrophage recruitment, and inflammatory Th1/Th17 T helper lymphocyte participation in the granulomatous response. The disease may spontaneously resolve or may progress to fibrosis, lung pathology and death. Regulatory T cells (Treg) are considered to restrain overt T helper cell activity and the extent of inflammation but if they are dysfunctional, disease progression occurs. Thus, the clinical picture closely resembles the experimental animal models. Further observations are needed to unravel Th-Treg interaction and the precise role of Treg before effective therapy can be devised.

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The linkage of innate and adaptive immune response during granulomatous development

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Granulomas represent a spectrum of inflammatory sequestration responses that may be initiated by a variety of agents, including non-infectious environmental factors and infectious microbial pathogens. Although this reaction is designed to be protective, the associated tissue injury is often responsible for a profound degree of pathology. While many of the mechanisms that sustain the development of the granuloma are enigmatic, it is accepted that the maintenance of this inflammatory process is dependent upon dynamic interactions between an inciting agent, inflammatory mediators, various immune and inflammatory cells, and structural cells of the involved tissue. The best studied of the host-dependent processes during granuloma development is the innate and adaptive immune response. The innate immune response by antigen-presenting cells [APCs; dendritic cells (DCs) and macrophages] is initiated quickly to protect from overwhelming pathogens, but with time, can also activate the adaptive immune response. APCs, essential regulators of the innate immune response, can respond to microbial ligands through Toll-like receptors (TLRs), which function in the recognition of microbial components and play an important role to link the innate and adaptive immune responses. $CD4^+$ T helper (Th) cells are essential regulators of adaptive immune responses and inflammatory diseases. Recently, the Notch system has been shown to be an important bridge between APCs and T cell communication circuits. In the present review, we discuss recent findings that explore the mechanisms in the linkage of innate and adaptive immunity, including granulomatous formation though TLRs and Notch activation.

Keywords: Notch signaling, Toll-like receptor, dendritic cell, T helper cell, innate immunity, acquired immunity

INTRODUCTION

The granulomatous response is a complex host defense mechanism that has evolved to provide containment of infectious and/or environmental agents (Warren, 1976; El-Zammar and Katzenstein, 2007). The maintenance of this inflammatory process is dependent upon an inciting agent and the dynamic interactions amongst inflammatory mediators, various immune and inflammatory cells, and structural cells of the involved tissue (Chensue et al., 1994; Ulrichs and Kaufmann, 2006; Ramakrishnan, 2012). The best studied of the host-dependent processes during granuloma development are the innate and adaptive immune responses, which are characterized by specific immune cell populations each expressing a defining phenotype (Chiu et al., 2004; Raymond et al., 2007; Wolf et al., 2007; Carson et al., 2011).

The initial response to infection is the engagement of the host's innate immune system that triggers a rapid, multifaceted anti-infectious response involving the release of proinflammatory cytokines and eventually leads to the activation of the adaptive immune response (Kumar et al., 2009). The first line of defense is initiated when cellular pattern recognition receptors (PRRs) located on antigen-presenting cells (APCs) recognize pathogen-associated molecular patterns (PAMPs) (Guillot et al., 2005; Goodman et al., 2010). Recognition of PAMPs by PRRs rapidly triggers the innate immune response characterized by an array of antimicrobial immune responses through the induction of various inflammatory cytokines and chemokines (Kumar et al., 2009, 2011). Several families of PRRs, including Toll-like receptors (TLRs), Retonoic acid-inducible gene I (RIG-I)-like receptors, nucleotide-binding oligomerization-like receptors, and DNA receptors (cytosolic sensors for DNA) are known to play a crucial role in host defense (Kumar et al., 2009, 2011). Dendritic cells (DCs) and macrophages are professional APCs that can respond to pathogens through PRRs, which function in the recognition of infectious components and play an important role in both the innate and adaptive immune responses (Akira et al., 2006; Trinchieri and Sher, 2007). Over time APCs can activate the adaptive immune response to the invading pathogens by triggering T cell differentiation (Lukacs et al., 2008; Cuddapah et al., 2010). Recent data have indicated that the controlled expression of Notch receptor proteins on T cells is essential for normal T cell development and maturation (Osborne and Minter, 2007). The connection between PRRs and Notch pathways has helped to define the complex role of APCs in the regulation of T cell differentiation (Amsen et al., 2009b). We here review recent advances concerning the role of Notch signaling in T cell differentiation

during infection and present our findings showing the role of the Notch system in linking innate and acquired immunity in inflammatory granulomatous response models.

TYPE-1 AND TYPE-2 GRANULOMA MODELS IN MICE

Data derived from a variety of models demonstrate that a number of inflammatory systems are involved in the induction of cytokines, which subsequently play a role in the initiation and maintenance of chronic experimental pulmonary inflammation (Raymond et al., 2007). However, the mechanistic contribution of various cytokines during the evolution, and more importantly, the maintenance of disease chronicity only recently have been addressed. For example, *in vivo* studies assessing the type-1 response elicited during the development of chronic lung granulomas induced by mycobacterial antigen have demonstrated that Interferon (IFN)-γ, Interleukin (IL)-12, and tumor necrosis factor (TNF) were necessary for lung lesion progression (Chensue et al., 1994). In contrast, type-2 experimental chronic lung granulomas initiated by complex antigens, including those derived from allergens (e.g., ovalbumin) and parasites (e.g., *Schistosoma mansoni*), were shown to be maintained by IL-4, IL-5, and IL-13, hallmarks of a type 2 response (Chensue et al., 1992; Ruth et al., 2000; Joshi et al., 2008). One of the most widespread and well-characterized type-1 granuloma diseases is tuberculosis. It has been estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*—resulting in more than 8.8 million cases of active tuberculosis with 1.4 million deaths globally in 2010 alone. We have established clinically relevant type 1 animal models with signature type-1 cytokine phenotypes. These models are established by the pre-sensitization of mice with either Mycobacterium species (BCG) or *Schistosoma mansoni* antigen followed by a pulmonary challenge with sized Sepharose beads coated with a known amount of tuberculin purified protein derivative (PPD) (Chensue et al., 1995; Ito et al., 2007, 2009a). We have also established a clinically relevant experimental type 2 model of inflammatory granuloma development by delivering *Schistosoma mansoni* eggs to the lungs, which release highly antigenic glycoproteins, referred to as Schistosoma egg antigen (SEA), that promote a dominant Th2 response (Chensue et al., 1992; Ruth et al., 2000; Ito et al., 2009b) (**Figure 1**). *Schistosoma mansoni* affects more than 80 million people worldwide, causing the disease intestinal schistosomiasis (Crompton, 1999; Hotez et al., 2008). It is the most widespread of the human-infecting schistosomes, and the host immune response to *S. mansoni* infection has been the most widely studied of the major schistosome species (Pearce and Macdonald, 2002; Schramm and Haas, 2010). These data offer the basis for continued studies to understand the mechanisms underlying these widespread diseases.

INNATE AND ACQUIRED IMMUNITY IN GRANULOMA DEVELOPMENT

The typical granuloma contains mostly macrophages and DCs, surrounded by T lymphocytes, while myeloid DCs also have been found in the granulomas of tuberculosis patients and mouse tuberculin models (Ulrichs and Kaufmann, 2006; Ito et al., 2007; Silva Miranda et al., 2012). Of these cells, macrophages are the dominant cell type found in granulomas (Pieters, 2008), and these can be of two varieties: classically activated (M1) macrophages and alternatively activated (M2) macrophages, each

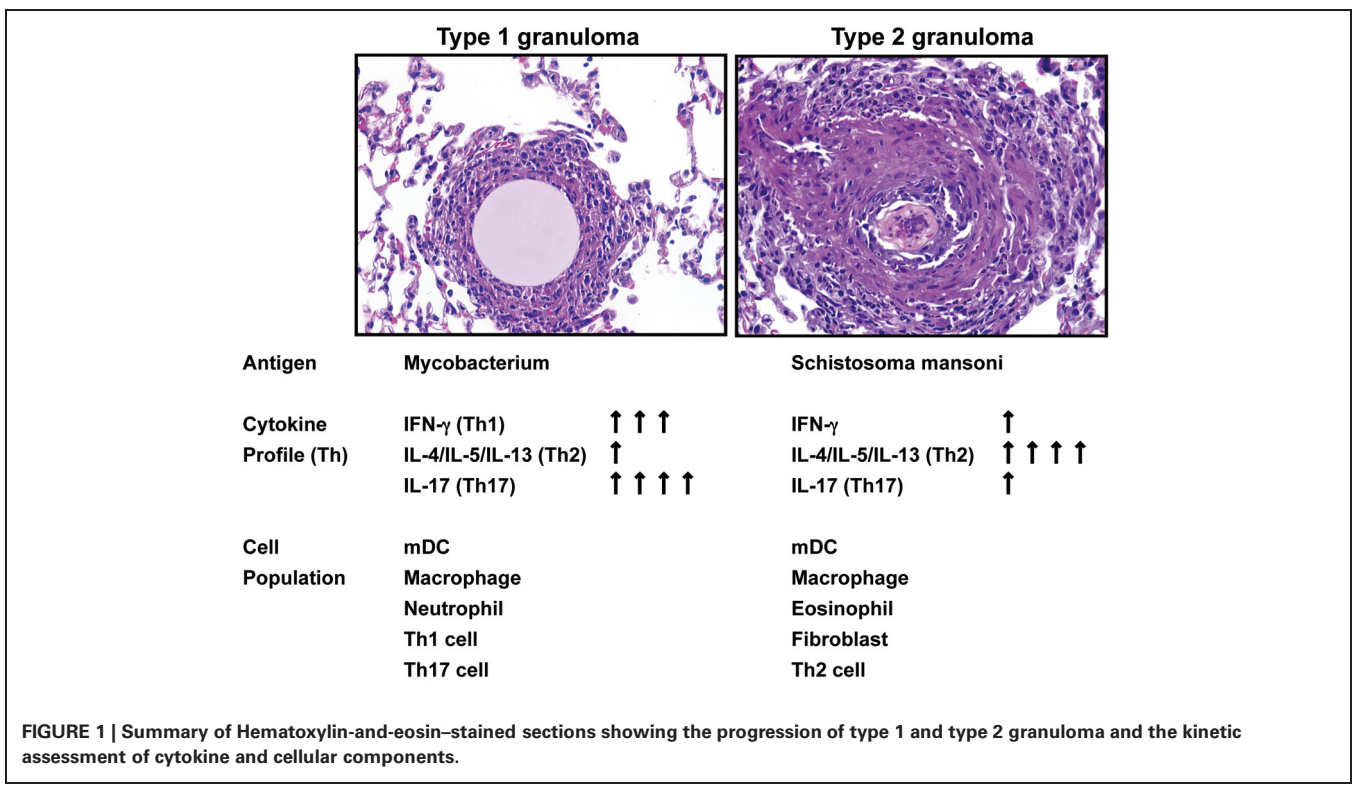


FIGURE 1 | Summary of Hematoxylin-and-eosin–stained sections showing the progression of type 1 and type 2 granuloma and the kinetic assessment of cytokine and cellular components.

of which have characteristic gene-expression profiles defined by markers linked to the stimulation conditions used to generate the subtype (Gordon and Taylor, 2005; Mantovani et al., 2007). Bacterial infection drives TLR engagement and IFN-γ expression skewing toward the M1 macrophage phenotype (pro-inflammatory and initiamicrobicidal), while *Schistosoma mansoni* infection drives IL-4 and IL-13 skewing toward the M2 macrophage phenotype (immunosuppressant and tissue repairers) (Herbert et al., 2004; Mosser and Edwards, 2008). In the granuloma, the presence of both types of macrophages may be required to maintain a balance between the pro- and anti-inflammatory response (Pieters, 2008). Although in comparison to macrophages, there are fewer numbers of DCs surrounding granulomas, the DCs play a critical role in antigen presentation, and produce large amounts of MHC-II and co-stimulatory molecules, initiating the linkage between innate and acquired immunity, which leads to T cell differentiation and activation (Wolf et al., 2007). T lymphocytes account for 15–50% of the leukocytes in mouse granulomas. About 60–70% of the T cells present are $CD4^+$, 15–30% are $CD8^+$ T cells, and there are also about 2%γδ T cells (Tsai et al., 2006; Silva Miranda et al., 2012). Of note, for example, the essential role of $CD4^+$ T cells in the control of mycobacterial infection has been highlighted by many studies in knockout mice, and also in HIV patients (Ladel et al., 1995; Ito et al., 2007, 2009a; Geldmacher et al., 2012; Silva Miranda et al., 2012).

TLRs FOR INNATE IMMUNITY

Innate immunity is the first line of host defense directed against invading pathogens and is designed to maintain host integrity (Si-Tahar et al., 2009). Innate immunity is activated immediately upon infection, is not antigen-specific, has no memory requirement, and requires large numbers of cells for pathogen recognition (Kabelitz and Medzhitov, 2007). Only if the invading pathogen is able to escape or overwhelm the innate response is acquired immunity activated. In some of these latter cases the host pro-inflammatory immune response becomes excessive, leading to pathological inflammation (Si-Tahar et al., 2009). Recent data suggest that the innate immune response initiated by the PAMP activation of specific TLRs can contribute mechanistically to maintaining the intensity and chronicity of the lung pathology associated with the developing granuloma (Ito et al., 2007, 2009b; Raymond et al., 2007). TLRs are named after their similarity to Toll, an essential receptor of the innate immune system against fungal infection, first discovered in Drosophila (Lemaitre et al., 1996). In recent years the family of mammalian TLRs expressed on APCs, namely DCs and macrophages, has been found to be key PRRs with central roles in the induction of the innate immune response. The principal functions of macrophages and DCs are phagocytosis and the elimination of microorganisms (Takeda and Akira, 2005). These cells also possess secondary functions including the production of cytokines, chemokines and chemotactic lipids, which direct certain circulating cells to migrate to the site of infection and participate in the elimination of pathogens. DCs also play an important role in communicating with and presenting antigens to lymphocytes, thus linking the innate and adaptive immune responses.

Microbial products, including mycobacterium antigen, activate specific TLRs, which in turn induce specific gene transcription resulting in the up-regulation and secretion of select chemokines and cytokines (Krutzik and Modlin, 2004; Ryffel et al., 2005; Jo et al., 2007). When activated, TLR specifically recruit adapter proteins [Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL), TIR domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM)], which are essential for the TLR gene transcription signaling cascade (Kawai and Akira, 2010; Kumar et al., 2011). Recent studies have provided new insights on how TLRs are involved in the recognition of specific pathogens, and have clarified their roles in both the innate and adaptive immune response (Goldstein, 2004). For example, mycobacterial components act as agonists for TLR, and mice that are deficient in the TLR adaptor molecule, MyD88, show an impaired response to mycobacterial antigens (Fremond et al., 2004). Although the response to mycobacterium antigens appears dependent on MyD88, the TLR involved has not been identified. All TLR, except TLR3, have at least one signaling pathway dependent on MyD88 (Kawai and Akira, 2010). The involvement of different TLRs, including TLR2, TLR4, TLR6, and TLR9 has been shown to recognize mycobacterium antigens in both mice and humans (Abel et al., 2002; Sugawara et al., 2003; Bafica et al., 2005; Motsinger-Reif et al., 2010). Specifically, TLR9 recognizes viral and bacterial CpG-DNA motifs, which when bound to TLR9 on macrophages and DCs, cause their activation (Jakob et al., 1998; Sparwasser et al., 1998; Hemmi et al., 2000). Further, TLR9 plays an important role in the regulation of the mycobacteria-induced T helper (Th) responses during *M. tuberculosis* infection *in vivo* (Bafica et al., 2005; Ito et al., 2007). The activation of TLR9 requires the uptake of microbes (or synthetic CpG oligodeoxynucleotides) within endosomes, the formation of DNA:TLR9 complexes within the endocytic vesicles, and the subsequent acidification and maturation of the endosomes (Latz et al., 2004; Wagner, 2004; Yasuda et al., 2005). Some observations support a role for TLR9 in the host response to lung infectious pneumonia induced by a variety of microbes including *Mycobacterium tuberculosis* (Bafica et al., 2005; Bhan et al., 2007; Kleinnijenhuis et al., 2011). We will return to this point later when we review our own recently published data.

NOTCH SYSTEM FOR ACQUIRED IMMUNITY

Pathogens such as bacteria, helminths, fungi, and viruses are recognized by and activate APCs, which in turn activate $CD4^+$ Th cells (Kumar et al., 2011). The Th cells drive adaptive immunity and induce specific responses against the infecting microbes (Trinchieri and Sher, 2007). It has been shown that the different types of APCs and their availability to display particular cytokine production profiles, pathogen recognition receptors (PRRs), and co-stimulatory molecules are key determinants for Th differentiation (Carballido et al., 2006).

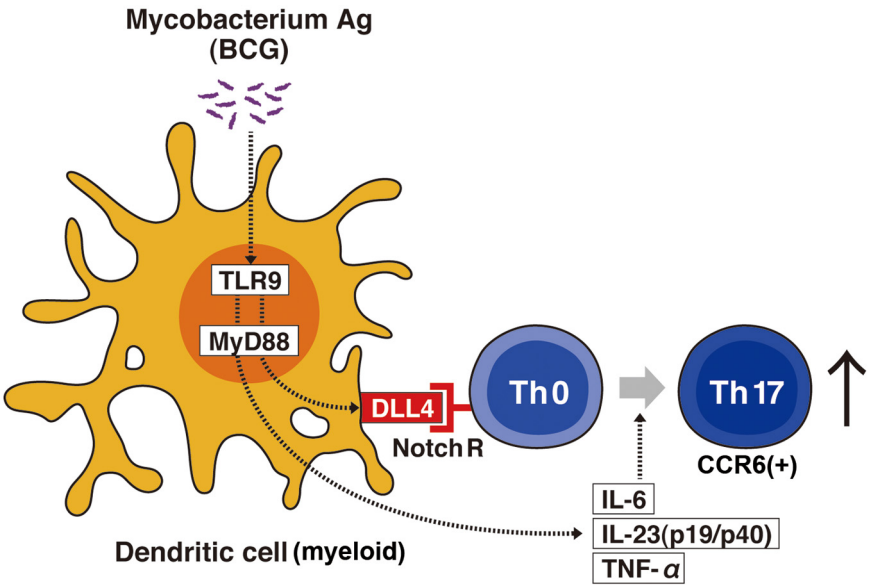
In addition, it has been shown that Notch proteins are also important in the induction of Th responses (Amsen et al., 2009a; Radtke et al., 2010). Notch is a heterodimeric cell-surface receptor family (Notch 1–4) that is involved in a broad range of differentiation processes (Bray, 2006). Notch family members are composed

of an extracellular ligand-binding domain that is non-covalently associated with a single-pass transmembrane domain. The Notch signaling pathway regulates many aspects of embryonic development, as well as differentiation processes and tissue homeostasis in multiple adult organ systems. There are two distinct families of Notch ligands in mammals, known as the Delta-like ligands (consisting of Dll1, Dll3, and Dll4) and the Jagged ligands (Jagged1 and Jagged2); both Dll and Jagged proteins trigger the canonical Notch signaling pathway wherein, binding of a ligand to a Notch receptor results in the cleavage of the receptor at a site in the transmembrane portion (Ehebauer et al., 2006). Upon binding by either Dll or Jagged ligands, Notch undergoes proteolytic cleavage catalyzed by Adam proteases and the γ-secretase complex, leading to the translocation of the notch intracellular domain (N-ICD) into the nucleus. N-ICD interacts with the transcriptional repressor, recombination-signal-binding protein for immunoglobulin-kJ region (RBP-J). The N-ICD interaction with RBP-J and also recruits Mastermind (MAML) protein. The new transcriptional complex of N-ICD-RBP-J-MAML converts RBP-J from a repressor to a transcriptional activator (Dallman et al., 2003; Ehebauer et al., 2006). Regulation of Notch signaling is associated with several human disorders, including cancer. It has been well documented in cancer studies that the Notch pathway influences stem cell maintenance, development and cell fate, and that it also promotes cell survival, angiogenesis, and treatment resistance in numerous cancers, making it a promising target for cancer therapy (D'Souza et al., 2008). More recently, it has become evident that Notch signaling plays an important role within the hematopoietic and immune systems. In the mature immune system, the Notch pathway has been shown to be involved in regulating Th1, Th2, and Th17 cell lineage choices for T cells, each of which is characterized by the production of distinct cytokines and effector functions (Radtke et al., 2010; Ito et al., 2012). For example, Th1 cells produce IFN-γ and target intracellular pathogens, while Th2 cells secrete IL-4, IL-5, and IL-13 and target helminthes (Kapsenberg, 2003; Ito et al., 2009b). In the presence of functional MyD88, an adaptor molecule of TLR, PAMP (derived from bacteria, viruses, or other TLR-ligands) binding to TLR upregulates Dll1 or Dll4 on APCs, which causes the differentiation of naïve Th cells to a Th1 phenotype (Maekawa et al., 2003; Amsen et al., 2004, 2009b). On the other hand, the differentiation of naïve Th cells to a Th2 phenotype occurred in the absence of functional MyD88 when Jagged was constitutively expressed on APCs (Amsen et al., 2009b). Thus data suggest that Dll1 and Dll4 cause Th1 skewing while Jagged causes Th2 skewing. Moreover, recent findings described IL-17-producing Th17 cells that play an essential role in host defense via protection against extracellular bacterial and fungus, while also recruiting neutrophils to the site of infection (Korn et al., 2009; Pappu et al., 2011). Neutrophils play a key role in the front-line defence against invading pathogens by phagocytosis as well as by recruiting other inflammatory cells (Lowe et al., 2012). Moreover, Th17 cells not only defend against bacterial and fungus infections but also are involved in autoimmune disease, allergic responses, and cancer (Wilke et al., 2011). However, how Dll- and Jagged-expressing APCs differ in their ability to induce Notch signaling and discriminate amongst their various described functions is unclear. More in-depth studies using mice that are deficient in each Notch and Notch ligand protein will provide data about how specific Notch ligands (Delta and Jagged ligands) control the different types of T cell responses during physiological conditions. Validation of the *in vitro* data will require a number of *in vivo* studies using diverse granulomatous models, including bacterial, parasitic, and fungal, as well as autoimmune models.

MYCOBACTERIAL GRANULOMATOUS RESPONSE THOUGH TLRs AND NOTCH ACTIVATION

While the mechanism of granuloma formation is unclear, this distinct cellular response is considered a histologic hallmark for a protective immune response, involving both innate and adaptive immunity. TLR9 is known to play a role in the regulation of Th1 responses (Bafica et al., 2005; Huang et al., 2005); thus, we have investigated the role of TLR9 in granuloma formation during challenge with mycobacterium antigens and demonstrated that mice deficient in TLR9 had increased granuloma formation with a dramatically altered cytokine phenotype. While Th1 cytokine levels of IFN-γ and IL-12 in the lungs were decreased in $TLR9^{-/-}$ mice when compared to wild-type mice, Th2 cytokine levels of IL-4, IL-5, and IL-13 were increased in these knockout mice (Ito et al., 2007). This response suggests that IL-12 production is TLR9-MyD88 pathway dependent. More recently, our group showed the first analysis of cell-mediated Th17-related pulmonary mycobacterial Ag-elicited granuloma formation in $TLR9^{-/-}$ mice and defined a role for TLR9 in the induction of both Notch ligand Dll4 and Th17 expression using both *in vivo* and *in vitro* approaches (Ito et al., 2009a). Our studies demonstrated that $TLR9^{-/-}$ mice exhibited significantly larger granuloma formation, following an impaired Th17-like response with decreased expression of Dll4 on DCs from $TLR9^{-/-}$ mice compared with WT mice. Dll4 was the primary Notch ligand upregulated by mycobacterial infection of DCs in WT mice. When Dll4 was specifically blocked *in vivo* using anti-Dll4 Ab during mycobacteria-induced pulmonary granuloma formation, Th17 cellular responses were significantly inhibited and larger granulomas were observed. Moreover, in *in vitro* experiments, anti-dll4 antibody specifically blocked IL-17 production by $CD4^+$ T cells, while overexpression of Dll4 augmented IL-17 production by $CD4^+$ T cells, suggesting that Dll4 plays an important role in promoting Th17 activity during a mycobacterial challenge. In contrast, the observed histologic alterations in lung granuloma development in $TLR9^{-/-}$ mice also coincided with a significant decrease in lung myeloid DCs, which are crucial to the differentiation of Th17 cells, as well as decreased levels of dll4 on DCs when compared with wild-type mice. This impaired migration of lung myeloid DCs in granuloma was attributed to decreased production of chemokine CCL20. Chemokines constitute a family of structurally related chemotactic cytokines that direct the migration of leukocytes throughout the body under both physiological and inflammatory conditions (Matsushima, 2000). CCL20 and its receptor CCR6 play a role in the recruitment of immature DCs and their precursors to sites of potential antigen entry (Schutyser et al., 2003). Thus, the lower expression of CCL20 during mycobacterial challenge in either TLR9-deficient mice or anti-dll4–treated lungs might contribute to the observed

decreased DC numbers during pulmonary granuloma formation. In addition, Th17 cells induced *in vivo* in normal mice via homeostatic proliferation express CCR6 as well as CCL20 (Hirota et al., 2007). Our data show that lower CCL20 expression in lungs from TLR9$^{-/-}$ mice and in lungs with anti-dll4 Ab is correlated with not only impaired DC migration but also reduced numbers of Th17 cells in lungs during mycobacteria induced pulmonary granuloma formation (Ito et al., 2009a) (**Figure 2**).



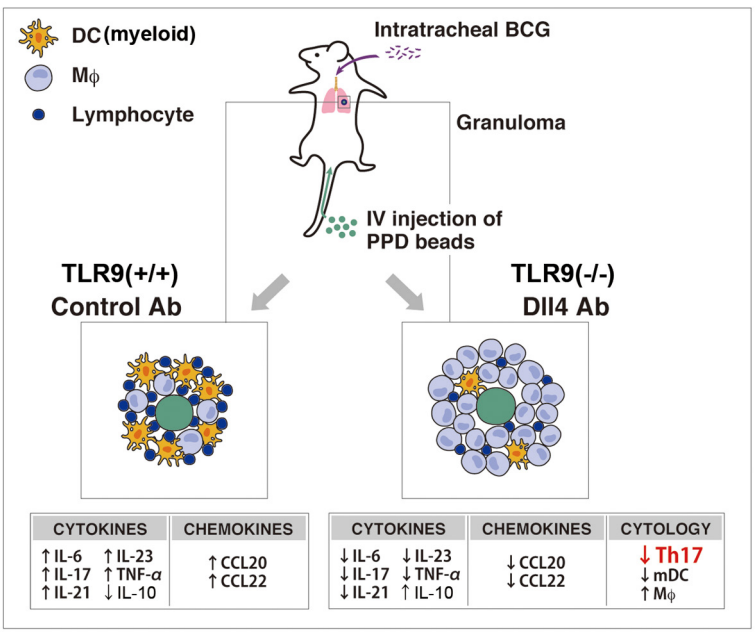


FIGURE 2 | Schematic representation of the TLR9-Notch ligand (dll4) on *Mycobacterium*-dependent granuloma formation. Myeloid DCs (mDCs) play an important role in inducing the differentiation of Th17 cells through the TLR9 effector pathway that upregulates the Notch ligand dll4. *In vivo* granuloma formation induced by BCG/*Mycobacterium* Ag demonstrates larger granuloma formation in TLR9-knockout mice (TLR9$^{-/-}$) with decreased numbers of Th17 cells (CCR6+) and mDCs in the lungs when compared with lung granulomas from WT mice. Further, TLR9$^{-/-}$ mice showed an increase in IL-10 with a concomitant decrease in Th17 cell-related cytokines (IL-17, IL-6, IL-21, IL-23, and TNF-α) and a decrease in the levels of the chemokines CCL20 and CCL22, important for DC migration, compared with levels in WT mice. The decreased expression of dll4 and the perturbation of the indicated cytokine and chemokine expression levels led to the abrogation of the Th17 phenotype in the Anti-Dll4 Ab treated mice with the concomitant increase in granuloma size. Accompanying these phenomena, there was a decrease in Th17 cells and mDCs in the lungs of Anti-Dll4 Ab treated mice and an increase in lung macrophages.

CONCLUDING REMARKS

Our data suggest that an understanding of Dll4 regulation of Th17 responses through Notch may provide mechanistic approaches for modifying and controlling the immune response induced by the Th17 phenotype. Moreover, a number of studies have demonstrated that Notch proteins are important in the induction of Th1 responses. In the presence of functional MyD88, PAMP binding to TLR up-regulates Dll4, which causes the differentiation of naïve Th cells to a Th1 phenotype. In addition, when Dll ligands are overexpressed on APCs or are cross-linked as fusion proteins, they also promote Th1 cell differentiation (Maekawa et al., 2003). Our recent study also revealed that Dll1 expression on macrophages is dependent on type-I IFN pathways, and is critical for protection against influenza virus A (H1N1) infection (Ito et al., 2011). Further, another of our recent findings indicated that Notch ligand Dll4 caused an increase in the expansion of Th2 memory cells and a decrease in effector cell proliferation in our experimental type 2 model of inflammatory granuloma development via the embolization of *Schistosoma mansoni* eggs to the lungs (Schaller et al., 2010). This study suggests that the Notch pathway also contributes to the different responses of memory and T effector (Teff) cells to Notch ligands, it also has been well established that Notch ligands can have different effects on T cell differentiation, depending on the immune environment including the local cytokine environment. However, the Notch system may be an even more complicated system, as these signaling pathways also contribute to multiple lineage decisions of developing lymphoid and myeloid cells. Moreover, the cellular constituents of the lung immune system are diverse and include not only leukocytes, such as macrophages, DCs, neutrophils, mast cells, and lymphocytes, but also, it has been shown that epithelial cells and fibroblasts play critical roles in the lung defense system. Further knowledge of the regulation of the Notch system in these cells and understanding the contextual interactions between these populations may provide mechanistic approaches for modifying and controlling the immune response during granulomatous diseases in clinically relevant translational studies. A better understanding of the regulation of the Notch system might contribute novel therapeutic approaches for granulomatous diseases.

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Chemokines in innate and adaptive granuloma formation

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Granulomas are cellular inflammations that vary widely in histologic appearance depending upon the inciting agent and immunologic status of the responding host. Despite their heterogeneity, granulomas are at their core an ancient innate sequestration response characterized by the accumulation of mononuclear phagocytes. In fact, this innate cellular response was first observed by Metchnikov in simple invertebrates. Among higher vertebrates, environmental pressures have resulted in the evolution of more sophisticated adaptive immune responses which can be superimposed upon and modify the character of granulomatous inflammation. Compared to immune responses that rapidly neutralize and eliminate infectious agents, the granuloma represents a less desirable "fall back" response which still has value to the host but can be co-opted by certain infectious agents and contribute to bystander organ damage. Understanding granulomas requires an analysis of the complex interplay of innate and adaptive molecular signals that govern the focal accumulation and activity of their cellular components. Among these signals, small molecular weight chemoattractant proteins known as chemokines are potentially important contributors as they participate in both directing leukocyte migration and function. This tract will discuss the contribution of chemokines to the development of innate and adaptive granuloma formation, as well as describe their relationship to more recently evolved cytokines generated during adaptive immune responses.

Keywords: granuloma, chemokines, chemokine receptors, innate immunity, inflammation

INTRODUCTION

The granuloma is defined as a cellular immune response in which the local accumulation of mononuclear phagocytes is a key component (Warren, 1976). The aggregation of mononuclear phagocytes is a sequestration response likely representing one of the earliest forms of innate cellular immunity. It is observed in primitive multicellular organisms and was first documented by Elie Metchnikov in his studies of starfish larvae. Over the millennia more sophisticated innate and adaptive immune responses have evolved, supplanting, or being superimposed upon the granuloma. In modern vertebrates, granulomas have varied histologic appearances and cellular elements depending upon the nature of the eliciting agent and host immune status, which determine the degree of cellular activity and chronicity of the response (**Figure 1**). In humans, there are many granulomatous conditions indicating that this response has persisted among the spectrum of immune responses and may confer some benefit, but compared to modern adaptive immune responses which can often rapidly eliminate microbes, the granuloma may be a "fall back" response relying on sequestration and attempted elimination. However, some pathogens such as *Mycobacterium tuberculosis* can co-opt the response residing for decades within granulomas only to reemerge when adaptive immunity is compromised. Other undesirable features of granulomas are chronic bystander organ damage and fibrosis. Hence, an understanding of the mechanisms underlying granuloma formation would potentially have important therapeutic benefit.

CHEMOKINES AND CHEMOKINE RECEPTORS

A basic requirement of granuloma formation involves directed cell migration to achieve focal cellular accumulation in response to infection, injury, or foreign bodies. It is well established that cell migration requires surface membrane receptors capable of recognizing extracellular molecules which initiate intracellular signal transduction events leading to selective gene expression and the cytoskeletal reorganization required for cell movement. Directed migration appears to depend upon cells sensing and following molecular chemotactic gradients of increasing concentration generated in the vicinity of an inciting agent. There are many types of chemotactic molecules but a family of homologous proteins with conserved motifs containing four cysteine residues known as chemokines, appear to be of particular importance. The topic of chemokines and their receptors has been extensively reviewed so need only a brief description herein (Zlotnik and Yoshie, 2012). There are over 40 known human chemokine ligands (L) divided among four classes based upon the arrangement of the amino terminal cysteine residues, these are suffixed, CCL, CXCL, CX3CL, and XCL. Most have homologs in the mouse and are produced by a wide variety of host cells. Their carbohydrate free molecular weight ranges from 7 to 10 kDa and they are often basically charged molecules with heparin binding properties. Their activity is mediated by seven-transmembrane domain, G protein-coupled receptors. The primary receptor-binding domain of all chemokines is near the extracellular amino terminus. Over 20 chemokine receptors have been characterized. Some display promiscuous ligand

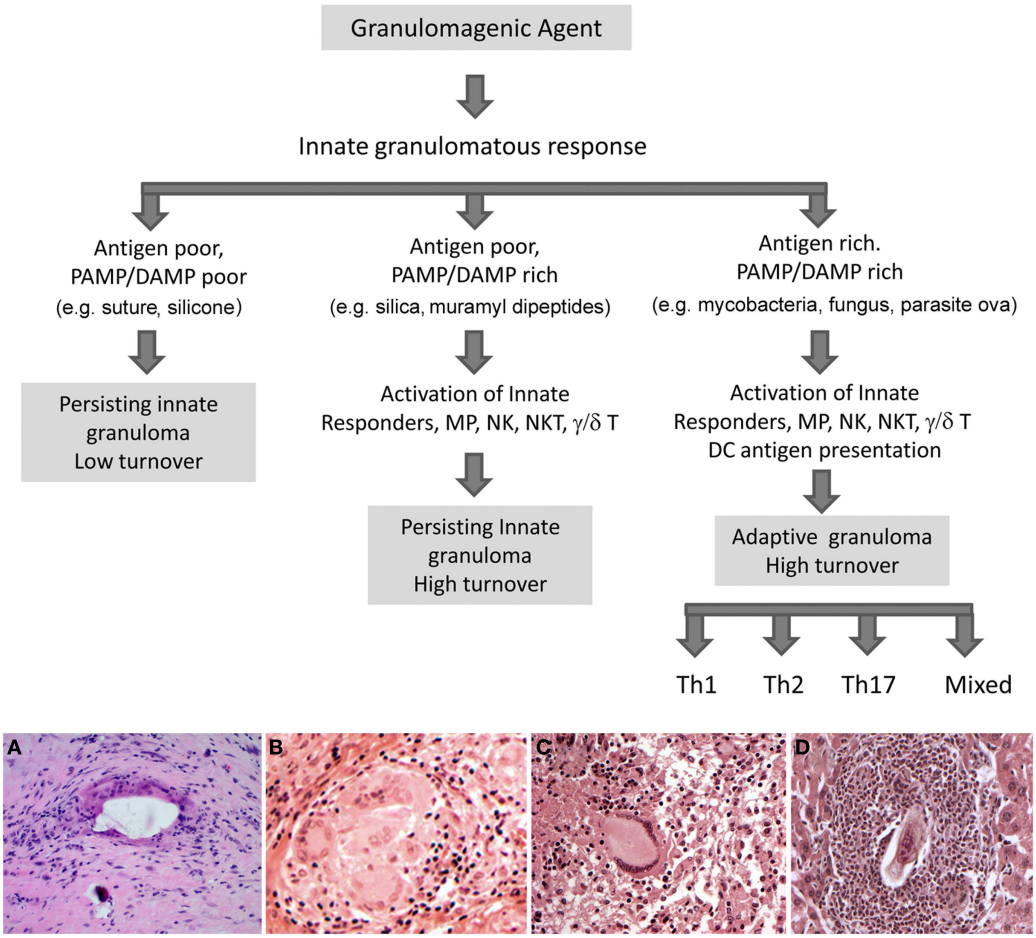

FIGURE 1 | Potential fates of the innate granuloma. In the immunocompetent host the ultimate type of granuloma manifested will depend in large part on the nature of the inciting agent. Weakly antigenic agents with poor expression of pathogen (PAMP) and or damage (DAMP) associated molecular patterns will result in a persisting low turnover innate granuloma. Examples would be surgical implant and suture granulomas. Weakly antigenic agents associated with greater PAMPs or DAMPs will activate a broader spectrum of innate responding cells resulting in more complex higher turnover innate granulomas. Examples would be silica, zymosan, and muramyl dipeptide induced granulomas. Strongly antigenic agents with associated PAMP or DAMP signals will initiate additional adaptive antigen-specific T cell responses resulting in a complex high turnover granuloma. The types of cellular components will depend upon the participating T cell subset. Examples would be mycobacterial, fungal, and parasite ova elicited granulomas. It should be noted that humoral elements including antibodies and complement may also influence lesions. Lower panels show examples of the histologic appearance of the various granulomas. **(A)** Innate low turnover foreign-body granulomatous response to surgical bone cement; **(B)** Innate high turnover granulomatous response to silica; **(C)** Adaptive granulomatous response to *M. tuberculosis*, Th1 type; **(D)** Adaptive eosinophil-rich, granulomatous response to schistosome parasite egg, Th2 type. Original magnifications 200×.

binding while others bind only a single known ligand. Receptor nomenclature is determined by the class of chemokine it binds (CCR, CXCR, CX3CR, and XCR). Receptors are differentially expressed by cell populations and can change during stages of cell maturation/activation. For example, CXCR1 and CXCR2 are highly expressed by neutrophils permitting responses to chemokine ligands CXCL1–8, which express the amino acid sequence of glutamic acid-leucine-arginine (ELR) immediately before the first cysteine of the CXC motif. In contrast, CXCR3 is expressed by effector lymphocytes and responds to CXCL9, 10, and 11, which lack the ELR sequence. Chemokines are expressed in a vast array of pathologic and physiologic states involving cellular migration. Therefore a mechanistic role in granuloma formation is a reasonable hypothesis. This tract will present a focused review of the studies performed in our laboratory to test this hypothesis.

EXPERIMENTAL GRANULOMA MODELS

In order to study mechanisms underlying granuloma formation, a number of animal-based experimental approaches have been developed which model a variety of granulomatous conditions, but due to widely varying experimental conditions it is difficult to compare cellular and molecular events among them. To provide a systematic experimental approach, our laboratory developed and characterized mouse models of synchronized lung granuloma formation elicited by intravenous embolization of agarose beads coupled to antigens derived from pathogens associated with

granulomatous diseases. Hence, granulomas elicited by diverse etiologic agents could be studied in parallel. As iconic representatives of highly prevalent granulomatous diseases, we utilized purified protein derivative (PPD) from *Mycobacterium bovis* and soluble egg antigens (SEA) derived from ova of the helminth parasite *Schistosoma mansoni*. Detailed immunologic characterization revealed that in pre-sensitized animals, the antigen-bead models respectively elicited polarized Th1 [interferon-γ (IFNγ) mediated] and Th2 (interleukin -4 and -13 mediated) adaptive hypersensitivity-type granulomas with cellular compositions similar to those elicited by live infections (Chensue et al., 1994). As well as being compared to each other, these adaptive responses could be compared to those elicited in unsensitized naive mice, representing the underlying innate granulomatous response. As shown in **Figures 2** and **3**, innate granulomas are smaller with comparable cellular compositions, whereas the adaptive responses are significantly larger with the PPD response showing greater neutrophil and NK cell component and the SEA response showing greater eosinophil content superimposed upon the large mononuclear infiltrate. The period of most rapid cellular recruitment occurs from 1 to 3 days with a period of sustenance followed by resolution.

CHEMOKINE EXPRESSION PATTERNS DURING INNATE AND ADAPTIVE GRANULOMA FORMATION

Using semiquantitative polymerase chain reaction gene expression analysis, we defined the temporal appearance of a panel of CXCL and CCL chemokines during synchronized innate and adaptive antigen bead-elicited granuloma formation (Chiu et al., 2003). Lungs of mice were harvested and analyzed at intervals after granuloma induction. As shown in **Figure 4**, both innate and adaptive responses displayed the greatest expression of chemokines in the first 2–3 days of granuloma formation, corresponding to the period of robust cellular recruitment. Despite quite different antigens, the patterns and degree of chemokines produced during innate granuloma formation in response to BSA, SEA, and PPD beads were very similar, except the PPD-elicited response showed higher expression of neutrophil chemotactins, CXCL2, CXCL5, and CCL3, which correlated with greater neutrophil recruitment. This finding suggested the presence of additional Ag-related innate recognition and was consistent with the ability of mycobacterial PPD components to stimulate Toll-like receptors, TLR2 and TLR4 (Means et al., 2001).

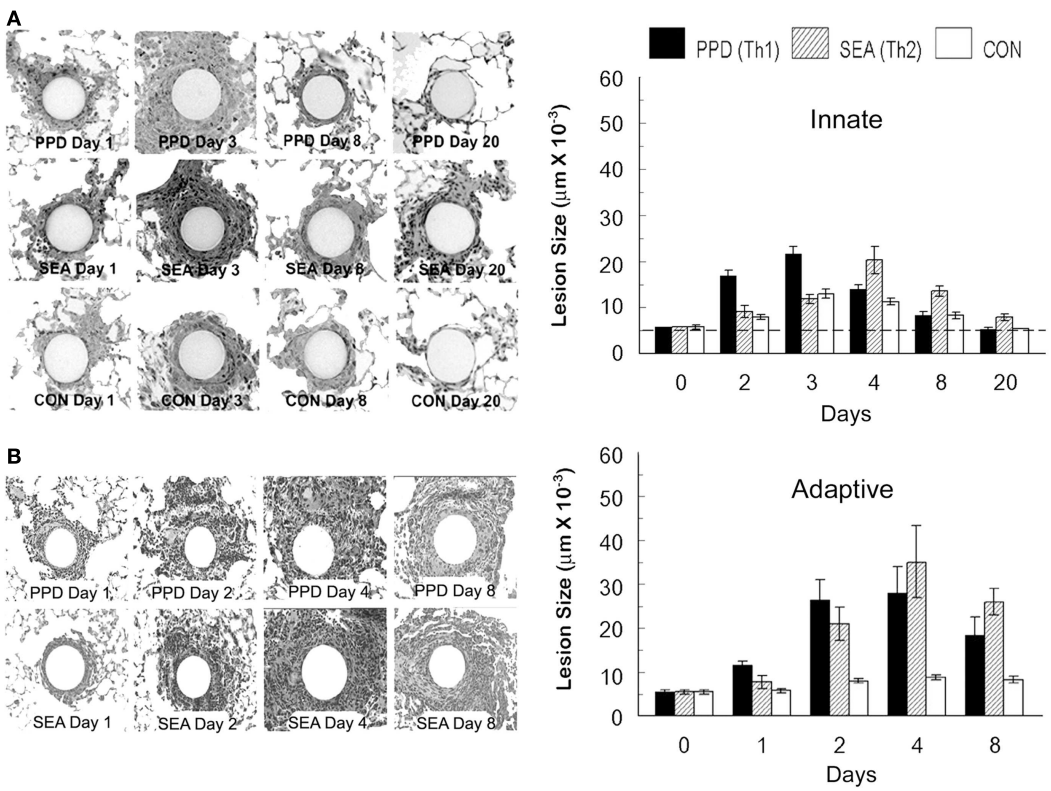

FIGURE 2 | Synchronized innate and adaptive antigen bead-elicited pulmonary granuloma formation. (A) Histologic appearance and sizes of innate antigen bead granulomas. Agarose beads covalently bound to *Mycobacterium bovis* purified protein derivative (PPD), *Schistosoma mansoni* soluble egg antigens (SEA), or bovine serum albumin (BSA), (CON) were administered intravenously to naïve C57BL/6 mice at a dose of 6000 beads per mouse. Lungs were harvested at the designated time points for histologic examination and cross-sectional measurement using computer assisted image analysis. A minimum of 20 lesions were measured. Dashed line represents average bead size. **(B)** Histologic appearance and sizes of adaptive antigen bead granulomas. Antigen beads were administered as above, but mice were pre-sensitized to the respective mycobacterial and schistosome egg antigens. Lungs were harvested at the designated time points for histologic examination and cross-sectional measurement as above. Size of a non-antigen coated control bead reaction is shown in chart for comparison. Bars are means and standard deviations of five mice.

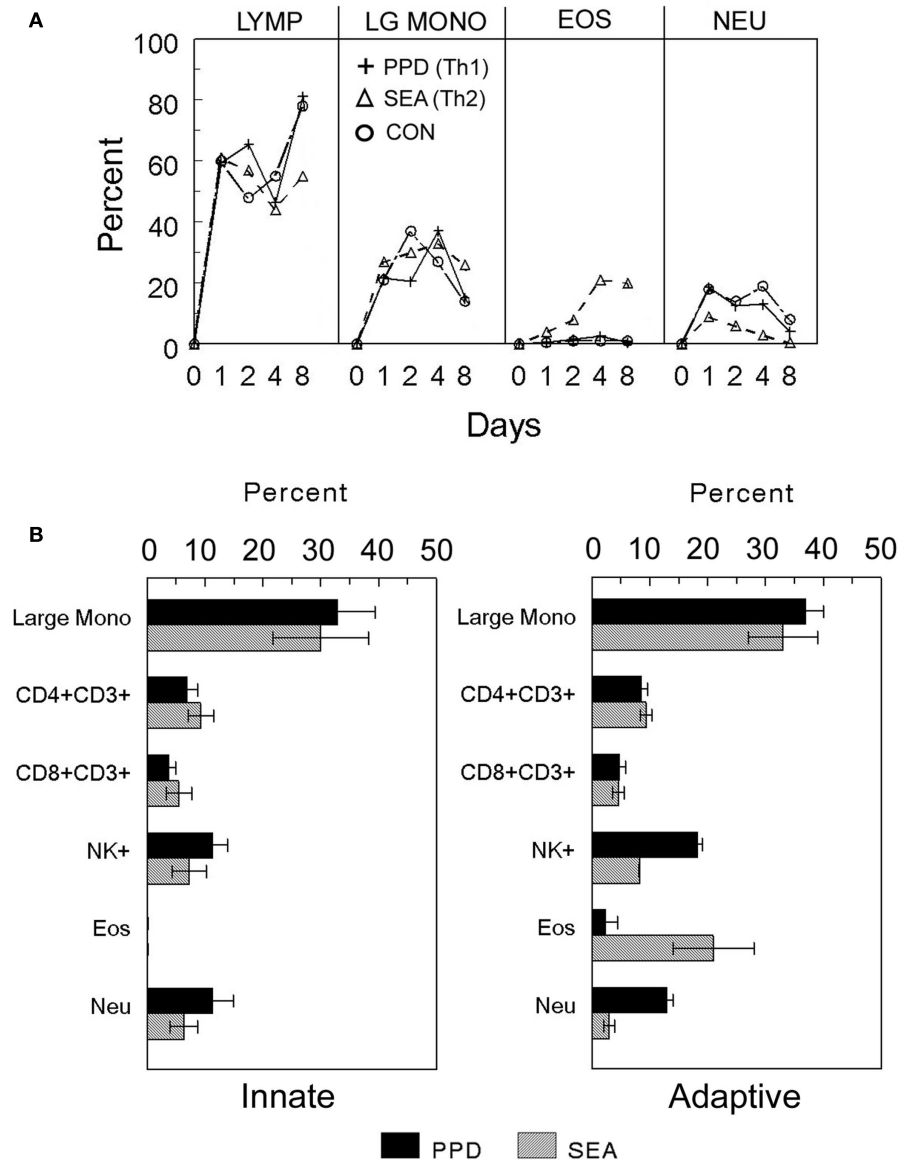

FIGURE 3 | Cellular composition of innate and adaptive antigen bead-elicited pulmonary granulomas. (A) Time course of major cell populations recruited during adaptive and innate granuloma induction. Innate BSA and adaptive PPD- and SEA-antigen bead lung granulomas were elicited as described in **Figure 2**. On the indicated days, granulomas were harvested and enzymatically dispersed, washed, and then used for differential analysis. **(B)** Day 4 cell composition of innate and adaptive PPD- and SEA-antigen bead granulomas. Granulomas were elicited and harvested as above and then washed cellular suspensions were subjected to differential analysis to determine cellular components. Lymphocytes (Lymp), large mononuclear cells (Large mono), eosinophils (Eos), and neutrophils (Neu) were determined by standard morphologic analysis. Flow cytometry was used to identify lymphoid subpopulations CD4+ and CD8+ T cells, natural killer (NK+). Bars are means and standard deviations of five mice.

During adaptive granuloma formation, polarization of the mycobacterial and helminth antigen bead-elicited chemokine profiles became clearly apparent. During PPD-bead elicited granuloma formation the CXCL chemokines dominated with levels increasing up to 10-fold over the innate response. In contrast, during the SEA response these remained at innate levels. Among the CCL chemokines, there was amplification of most but with a lesser degree of polarization. However, the SEA-bead elicited response displayed augmented CCL7 expression whereas the PPD response remained at innate levels. CCL7 is a ligand for the CCR3 receptor expressed by eosinophils which are a major component of the adaptive granulomatous response to SEA.

CYTOKINE REGULATION OF CHEMOKINE EXPRESSION DURING ADAPTIVE GRANULOMA FORMATION

Chemokine expression analyses of Ag-bead-elicited granuloma formation demonstrated the presence of innate chemokine responses which were modified by superimposition of adaptive

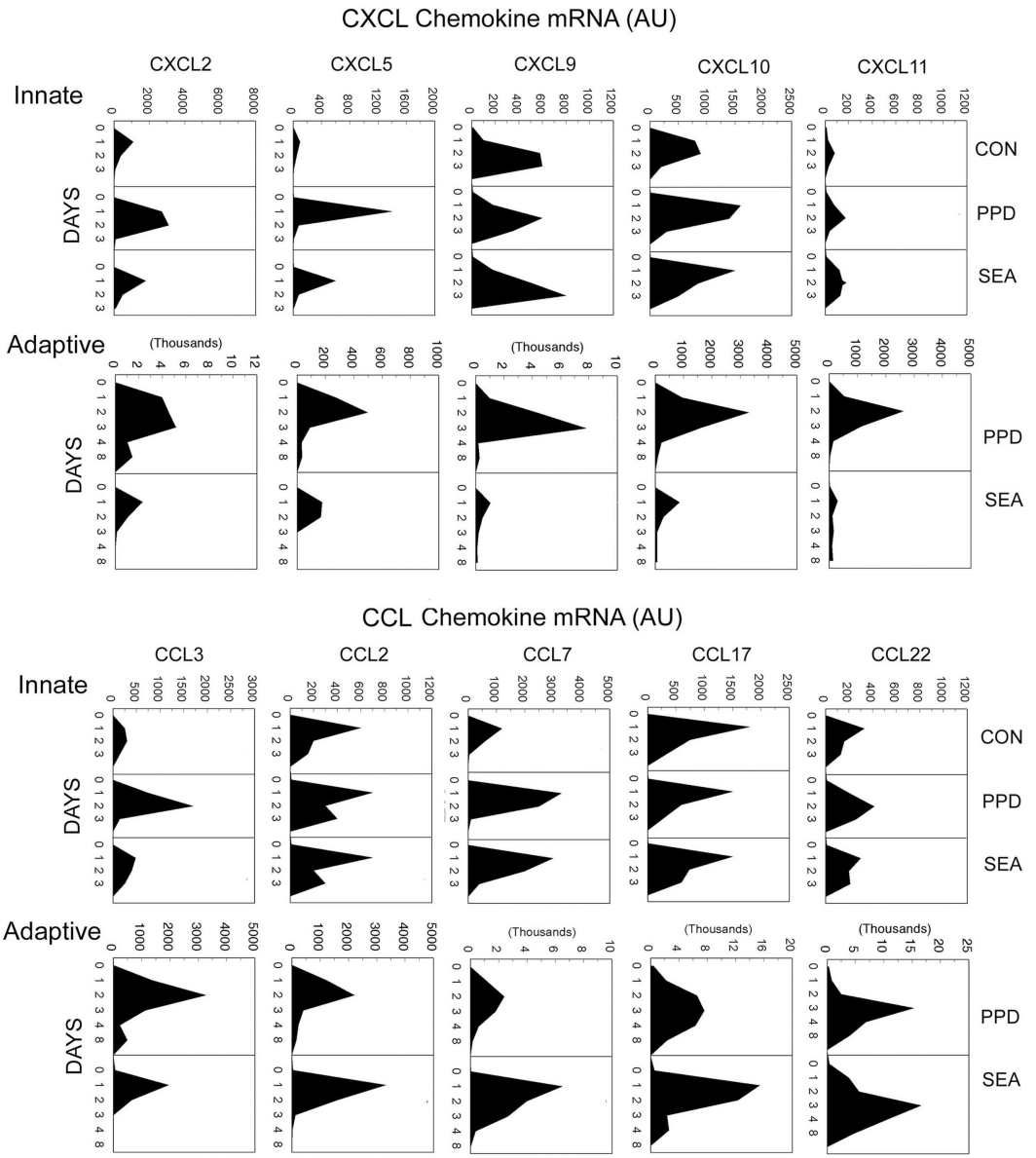

FIGURE 4 | Dynamics of chemokine transcript expression during innate and adaptive antigen bead-elicited granulomas formation. Innate and adaptive PPD- and SEA-antigen bead granulomas were elicited as described in **Figure 2**. Lungs were harvested at the designated time points for mRNA extraction and cDNA preparation. Chemokine transcript levels were determined by semiquantitative real-time RT-PCR. Mean arbitrary units (AU) were derived from five to six individual lungs per point.

immunity. Moreover, the character of the adaptive chemokine profile differed depending on whether the adaptive cellular response was either Th1- or Th2-mediated. To directly test if Th1 and Th2-related cytokines were responsible for shaping the chemokine patterns observed during the adaptive response, mice with either type-1 PPD- or type-2 SEA-bead elicited adaptive granulomas were treated with cytokine neutralizing antibodies to IFNγ, IL-12, IL-4, IL-10, or IL-13, then chemokine transcripts were measured on day 2, during the period of most active chemokine induction. As summarized in **Table 1**, IFNγ depletion decreased a number of the transcripts during mycobacterial PPD-elicited type-1 granuloma formation, especially the CXCL chemokines. This was associated with a significant reduction in granuloma size measured on day 4. In the helminth SEA-elicited type-2 response, the IFNγ-dependent CXCL9–11 chemokines were also reduced but CCL4, CCL7, CCL17, and CCL22 were increased with about a 30% augmentation of granuloma cross-sectional area. This demonstrated a cross-regulatory effect of endogenous IFNγ in the type-2 response. Anti-IL-12 also reduced CXCL9-11 during the type-1 response but with a lesser reduction in granuloma size. Since IL-12 is an innate stage cytokine known to enhance IFNγ, this likely represented an effect on the IL-12 dependent

Table 1 | Effect of cytokine depletion of chemokine transcript expression and granuloma formation*.

Granuloma type and treatment	CXCL2	CXCL5	CXCL9	CXCL10	CXCL11	CCL1	CCL2	CCL3	CCL4	CCL7	CCL8	CCL11	CCL17	CCL22	XCL1	Effect on lesion area
TYPE-1 (PPD)																
Anti-IFN																−41%
Anti-IL-12																−14%
Anti-IL-4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Anti-IL-13																
Anti-IL-10																
TYPE-2 (SEA)																
Anti-IFN																+28%
Anti-IL-12																
Anti-IL-4																−38%
Anti-IL-13																−30%
Anti-IL-10																

**Cells shaded red indicates significant decreases; green indicates significant increases and white indicates no change. ND, not determined.*

component of IFNγ production, though a direct effect could not be ruled out. In the type-2 response, IL-12 depletion had no effect on CXC but increased a number of the CC chemokines without affecting overall inflammation, suggesting that endogenous IL-12 has a regulatory effect but the enhanced CC chemokine transcript expression could not further augment inflammation. Since IFNγ depletion did reduce CXC chemokines and augmented type-2 inflammation, it suggests a role for CXC chemokines in recruitment or stimulation of a population that tempers type-2 inflammation. In the type-2 response, depletion of the Th2 cytokines, IL-4, or IL-3, caused reductions of most CC with no effect on the CXC chemokine transcripts, which was associated with significant reductions in granuloma sizes. Anti-IL-13 also reduced some CC chemokines in the type-1 PPD response with augmentation of CXCL9-11, indicating a regulatory effect of underlying IL-13, but no net effect on inflammation was observed. Depletion of endogenous IL-10 which may derive from both innate and adaptive immune cells appeared to influence only a limited number of CC chemokines with no effect on overall inflammation.

It should be noted that in addition to our finding of chemokine modulation by Th1- and Th2-related cytokines, recent studies of neutrophil-rich granulomas elicited by *S. japonicum* eggs show participation of Th17 cells and IL-17 dependence of neutrophil chemotactins, CXCL1, and CXCL2 (Zhang et al., 2012).

To summarize, cytokines appear to shape the spectrum and degree of chemokines produced during adaptive granuloma formation through positive and negative signaling. Polarized cytokine responses lead to different chemokine profiles presumably designed to generate a pathogen specific granulomatous response.

CHEMOKINE CONTRIBUTION TO GRANULOMA FORMATION

Published literature demonstrates consistent circumstantial expression of chemokines in animals models of granulomatous conditions and human granulomatous diseases. However, revealing their functional significance is more challenging. Because, of their potential promiscuity in receptor-binding, experiments designed to neutralize or knockout individual chemokines leave open the possibility of compensatory or redundant chemokines. The alternative approach is to focus on the relative requirement of chemokine receptors, which are less numerous than chemokines and often show restricted cellular expression. An important aspect of chemokine receptors is their dynamic nature. A particular cell may express different receptors at different maturational stages as well as alter levels of expression following activation or receptor ligation. Most studies of chemokine receptors employ chemokine receptor knockout mouse strains or specific receptor antagonists. We and others have used these approaches to assess their role in granuloma formation. Those findings are discussed below.

CCR1

CCR1 (CD191) is a highly promiscuous receptor with reported binding to multiple CC ligands (CCL3, CCL5, CCL7, CCL8, CCL14, CCL15, CCL16, and CCL23). It is expressed by a number of cell types including macrophages and lymphocyte populations. When type-1 PPD- or type-2 SEA-bead elicited adaptive granulomas were elicited in CCR1 knockout ($CCR1^{-/-}$) mice, there was little effect on overall sizes of secondary lesions. However, there were subtle but distinct effects on the type-1 response. The latter unlike the type-2 lesion is characterized by a component of natural killer (NK) cells, which was absent in $CCR1^{-/-}$ mice. In addition, there was reduced production of Th1-associated cytokines by draining lymph node cultures (Shang et al., 2000). In a separate study, Gao et al. (2000) reported impairment neutrophil recruitment and abrogation of the granulomatous response to primary injection of whole *S. mansoni* eggs in $CCR1^{-/-}$ mice. When we tested the innate granulomatous response to PPD- and SEA-beads, we again showed impaired NK cell recruitment as well as compromised neutrophil recruitment (Chiu et al., 2004). Taken together, the findings suggest a role for CCR1 in the innate response, which may partially influence the quality of subsequent adaptive responses.

CCR2

Like CCR1, CCR2 (CD192) is a promiscuous receptor, binding CCL2, CCL7, CCL8, CCL13, and CCL16. A wide variety of cell types are reported to express CCR2 including monocytes, lymphocytes, and non-leukocytic cells. When type-1 PPD- or type-2 SEA-bead elicited adaptive granulomas were elicited in CCR2 knockout ($CCR2^{-/-}$) mice, there was impaired monocyte/macrophage recruitment especially during the initial stages of granuloma formation. In the type-1 response this was associated with notable reductions in Th1 cytokine production by draining lymph node cultures and while there was partial reduction of IL-4 producing capacity in the type-2 response the Th2 cytokine profile remained largely intact (Boring et al., 1997; Warmington et al., 1999). An analysis of innate granuloma formation in $CCR2^{-/-}$ mice similarly showed impaired monocyte/macrophage recruitment in both PPD- and SEA-bead elicited lesions. In addition, both showed significant reductions in granuloma associated myeloid dendritic cells (mDCs), thus providing a potential link between recruitment and adaptive phase cytokine defects (Chiu et al., 2004). A similar monocyte/macrophage recruitment defect has been reported in a model of zymosan-elicited granuloma formation (Jinnouchi et al., 2003). Together the findings suggest a critical role for CCR2 in the mobilization of the innate monocyte/macrophage and mDC component of granulomas. The lack of which can significantly influence subsequent adaptive stage events, mainly the Th1 response.

CCR3

CCR3 (CD193) is another promiscuous receptor binding CCL3, CCL5, CCL7, CCL11, CCL13, CCL14, CCL15, CCL24, and CCL26. It is highly expressed by eosinophils and basophils as well as lymphocyte subpopulations and some non-leukocytic cells. No systematic studies of granuloma formation in CCR3 knockout mice have been reported. However, we have tested the role of two its ligands, CCL7 and CCL11 (eotaxin). Transcript analyses in those studies showed induction of CCR3, CCL7, and CCL11 during both type-1 PPD- and type-2 SEA-bead elicited adaptive granuloma formation, but expression of all was greater in the latter and augmented expression was dependent on IL-4. *In vivo* CCL7 and CCL11 depletions revealed that CCL7 and not CCL11 contributed to eosinophil recruitment in the type-2 response (Ruth et al., 1998; Shang et al., 2002). These data provided indirect evidence of differential participation of CCR3 agonists and demonstrated a role for selected chemokines in shaping granuloma cellular composition.

CCR4

CCR4 (CD194) has two known agonist ligands CCL17 and CCL22. The receptor is reportedly expressed by a variety of leukocytic and non-leukocytic populations. Among T cells it has been mainly associated with skin homing CD4+ effector memory, Th2 effector, and T regulatory cells. Constitutive expression of CCL17 and CCL22 is detectable among myeloid DCs and is upregulated by inflammatory stimuli (Penna et al., 2002). As described above, these ligands are induced during innate and adaptive type-1 PPD- and type-2 SEA-bead elicited granuloma formation. When adaptive granulomas were elicited in CCR4 knockout ($CCR4^{-/-}$) mice, there was partial abrogation of the type-1 response and decreased local IFNγ production. The inflammation associated with type-2 granulomas was largely unaffected but showed reductions of local IL-5 and IL-13 production (Freeman et al., 2006). These studies indicated that in the mouse CCR4 participation was not restricted to Th2 dominant responses. Additional *in vivo* and *in vitro* experiments suggested that CCR4 was required for effector memory cell activation by mDCs. Thus, CCR4 may allow for optimal contacts between effector cells and mDCs under homeostatic and challenge conditions.

CCR5

CCR5 (CD195) is a promiscuous receptor-binding CCL4, CCL5, CCL8, CCL11, CCL14, and CCL16 as agonists. It has been extensively studied due to its ability to act a coreceptor for human immunodeficiency virus (HIV). CCR5 is expressed on T cells, macrophages, mDCs, and some non-leukocytic cells. Among T cells it reportedly shows biased expression by CD4+ Th1 and CD8+ T cells, however most reports suggest CCR5 is dispensable for mounting resistance to variety of pathogen challenges. In regard to granuloma formation, we observed no effect of CCR5 knockout on the innate response to either PPD- or SEA-beads (Chiu et al., 2004). However, Souza et al. (2011) demonstrated significant exacerbation of the Th2-mediated granulomatous response to *S. mansoni* eggs. This observation would be consistent with our finding that depletion of CCL5, a potent agonist of CCR5, augments type-2 granuloma formation (Chensue et al., 1999). Taken together, these studies suggest the presence of CCR5-dependent immunoregulation of type-2 adaptive granuloma formation.

CCR6

Unlike other CC chemokine receptors, the gene for CCR6 (CD196) is carried on a different chromosome (human chromosome 6, mouse chromosome 17) than the others which are clustered (human chromosome 3, mouse chromosome 9). It is also unique in that it has only one known chemokine agonist ligand, CCL20. CCR6 is expressed by mDCs, mature B cells, subpopulations of memory/effector CD4+ and CD8+ T cells, but not plasmacytoid DCs or naïve T cells. Among CD4+ T cells, CCR6 has been associated with Th1 and Th17 subpopulations. While the ligand for CCR6 has been reported in models of granuloma formation (Qiu et al., 2001), there are as yet no systematic functional analyses. Despite its expression by immature mDCs, no defect in local mDC recruitment was observed during the innate granulomatous response to PPD- or SEA-beads elicited in CCR6 knockout ($CCR6^{-/-}$) mice (Chiu et al., 2004). In preliminary studies of adaptive type-1 PPD- and type-2 SEA-bead elicited granuloma formation, $CCR6^{-/-}$ mice displayed no defects in type-1 inflammation and only a partial impairment of eosinophil recruitment in the type-2 response. However, alterations in draining lymph node cytokine and immunoglobulin induction events were noted (unpublished observations). A number of studies point to a role for CCR6 in mucosal and lymphoid tissue events (Ito et al., 2011), hence there may be less contribution to interstitial granulomatous responses. However, CCR6-mediated effects on the

adaptive immunity could influence some aspects of granuloma formation.

CCR8

CCR8 (CDw198) has a limited agonist complement, CCL1, CCL8 (mouse), CCL16, and is expressed by T cells and possibly macrophage subsets. Among T cells it has been associated with Th2 and T regulatory populations. There are limited studies examining the contribution of CCR8 to granuloma formation. We performed detailed analyses of type-1 PPD- and type-2 SEA-bead elicited adaptive granuloma formation in CCR8 knockout ($CCR8^{-/-}$) mice (Chensue et al., 2001; Freeman et al., 2005). Those studies showed the CCR8 was required for establishment of a normal eosinophil-rich type-2 granuloma, but was not required for the type-1 granulomatous response. The critical CCR8+ population was identified as a CD4+ IL-10+ regulatory T cell that appeared to enhance expression of the Th2 response likely by tempering cross-regulatory signals.

CXCR4

CXCR4 (CD184) has one known chemokine agonist, CXCL12, and is expressed by a variety of leukocytes including T cells as well as non-leukocytic cells. In addition to T cell chemotaxis, CXCR4 appears to play roles in regulating stem cell homing to the bone marrow, HIV entry into cells and tumor metastasis. Since CXCR4 knockout is lethal, we used a specific CXCR4 receptor antagonist, AMD3465, to test its effects on type-1 PPD- and type-2 SEA-bead elicited granuloma formation (Hu et al., 2006). CXCL12 transcripts are induced locally and in draining lymph nodes upon granuloma elicitation with the greatest expression in the adaptive type-2 response. Accordingly, treatment had no effect on the innate or the adaptive type-1 responses, but the type-2 adaptive response was significantly abrogated with reductions of both eosinophils and mononuclear phagocytes. In addition, Th2 cytokine production by draining lymph node cultures was notably compromised. Thus, CXCR4 showed biased participation in establishing the type-2 adaptive granulomatous response. Precise mechanisms remain to be determined.

CONCLUSION

Using models of synchronized innate and adaptive pulmonary granuloma formation we have demonstrated a role for chemokines in the development of these lesions. The emerging picture is one in which different chemokines contribute to different stages, components, and functional aspects of granulomas. Conceptualizing and classifying granulomas in terms of innate and adaptive responses allows for more precise interpretation of cellular events. As described above, some chemokines contribute more to the innate component of the response whereas others are invoked by adaptive stage molecular signals amplifying and altering granuloma cellular composition to provide functions needed to contain and or eliminate an insulting agent. As major mediators of the inflammation, chemokines are a potential target for therapeutic manipulations to temper or optimize the granulomatous response.

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The granuloma in tuberculosis: dynamics of a host–pathogen collusion

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A granuloma is defined as an inflammatory mononuclear cell infiltrate that, while capable of limiting growth of *Mycobacterium tuberculosis*, also provides a survival niche from which the bacteria may disseminate. The tuberculosis lesion is highly dynamic and shaped by both, immune response elements and the pathogen. In the granuloma, *M. tuberculosis* switches to a non-replicating but energy-generating life style whose detailed molecular characterization can identify novel targets for chemotherapy. To secure transmission to a new host, *M. tuberculosis* has evolved to drive T cell immunity to the point that necrotizing granulomas leak into bronchial cavities to facilitate expectoration of bacilli. From an evolutionary perspective it is therefore questionable whether vaccination and immunity enhancing strategies that merely mimic the natural immune response directed against *M. tuberculosis* infection can overcome pulmonary tuberculosis in the adult population. Juxtaposition of molecular pathology and immunology with microbial physiology and the use of novel imaging approaches afford an integrative view of the granuloma's contribution to the life cycle of *M. tuberculosis*. This review revisits the different input of innate and adaptive immunity in granuloma biogenesis, with a focus on the co-evolutionary forces that redirect immune responses also to the benefit of the pathogen, i.e., its survival, propagation, and transmission.

Keywords: granuloma, tuberculosis, pulmonary, life cycle stages, immunopathology, evolution

INTRODUCTION

The pathogenesis of tuberculosis used to be the investigative domain of two relatively separate, albeit complementary disciplines. On the one hand, molecular microbiologists decoded the survival strategies of *Mycobacterium tuberculosis* (*M. tuberculosis*), i.e., physiological and metabolic adaptation to the host environment, dynamics of replication, and synthesis and structures of the cell wall in order to define microbial factors important for virulence and persistence. On the other hand, infection immunologists analyzed innate and adaptive immune responses required to contain *M. tuberculosis* growth and dissemination, often welcoming the pathologist's view on granuloma initiation, maintenance, and disintegration. In recent years, a more integrated view of tuberculosis pathogenesis has prevailed: the granuloma is not only recognized as a tissue reaction to limit bacillary growth and sequester infection but also as part of the successful life cycle of *M. tuberculosis*, thus representing the dynamic combat zone between both, the pathogen and host defense elements. This co-evolutionary perspective emphasizes the mutual shaping of the tissue microenvironment, which, at the same time, allows propagation and transmission of *M. tuberculosis*, yet restricts tissue damage to safeguard survival of the host. This review will highlight recent findings that have shifted the long-held paradigm that in TB the granuloma is primarily or uniquely relevant for protection of the host. This integrative model of the granuloma's function in TB pathogenesis also challenges the concept that granulomas exclusively serve the interest of the pathogen.

INCIPIENT GRANULOMAS: FERTILE SOIL FOR MYCOBACTERIAL REPLICATION

After aerosol inhalation, the first host cell *M. tuberculosis* encounters is the alveolar macrophage, which phagocytoses but fails to kill the mycobacterial invaders, but does produce chemoattractants. Chemokines produced by alveolar macrophage and pneumocytes attract the first round of inflammatory cells, i.e., neutrophils, monocyte derived macrophages, NK cells, and γδ-T cells, which further promote inflammation and tissue remodeling (Feng et al., 2006; Lockhart et al., 2006; Eum et al., 2010). In mouse models of aerosol infection with mycobacteria, granuloma formation proceeds in the complete absence of specific immunity (North and Izzo, 1993; Hänsch et al., 1996; Smith et al., 1997). While TNF-α and IFN-γ accelerate inflammatory cell infiltration, they are not essential for granuloma initiation (Flynn et al., 1995; Smith et al., 1997). Non-activated macrophages, however, serve as feeder cells within the nascent granuloma (Davis and Ramakrishnan, 2009; Ehlers, 2010). Indeed, in the transparent zebrafish embryo model of *M. marinum* infection, a single virulence factor, which is present also in *M. tuberculosis*, ESAT6, is sufficient to induce matrix metalloprotease 9 production in epithelial cells (Volkman et al., 2010). This results in the recruitment of resting macrophages in which *M. marinum* replicates, and which even function as vectors that

spread infection to other body tissues (Davis and Ramakrishnan, 2009). In sum, early innate responses to *M. tuberculosis* infection do little to restrict and much to promote *M. tuberculosis* replication. Consequently, a focal accumulation of mononuclear cells in various states of differentiation, i.e., the initial stage of a granuloma, is not *per se* protective. Therefore it is no big surprise that the lack of the innate Toll-like or NOD-like receptors in mice, though involved in recognition of mycobacteria and subsequent induction of inflammation, has no major impact on the course of aerosol *M. tuberculosis* infection (Gandotra et al., 2007; Reiling et al., 2007; Walter et al., 2010). Similarly, C-type lectins including mannose receptor, CD38, DC-SIGN, or MINCLE, all recognizing various mycobacterial cell wall glycolipids, do not contribute much to protection, most probably due to a high degree of redundancy between those receptors (Court et al., 2010; Behler et al., 2012; Heitmann et al., 2012). It should however be mentioned that the mycobacterial glycolipid ligand of Mincle, trehalose dimycolate, alone is sufficient to induce inflammation and granuloma-like structures upon injection into mice, which is diminished in Mincle KO mice (Geisel et al., 2005; Ishikawa et al., 2009; Schoenen et al., 2010; Lee et al., 2012). Trehalose dimycolate, a mycobacterial virulence factor on its own right, may therefore represent a driving force in granuloma formation.

MATURE GRANULOMAS: DYNAMIC HETEROGENEITY

Following migration from the initial inflammatory focus to the regional lymph nodes, dendritic cells prime T cells for differentiation into predominantly TH1 and TH17 as well as cytotoxic T effector cells (Cooper, 2009). Recirculating primed pathogen specific T cells are critical for activating macrophages and curtail mycobacterial growth within the nascent granulomatous lesion (North and Jung, 2004). In the presence of activated T cells, the granuloma becomes fully organized, with mycobacteria-harboring macrophages at the center surrounded by a rim of lymphocytes. The ensuing stale-mate between host and pathogen, however, is much more dynamic than previously thought, and involves continuous loss of cells by cell death and replenishment by cellular recruitment, as well as vascular and tissue remodeling (**Figure 1**). Apart from inflammatory immune cells also mesenchymal stem cells are recruited, which seem to promote infection (Raghuvanshi et al., 2010). Pathologists have long been enamored with describing tuberculosis lesions as "exsudative, suppurative, miliary, caseous, circumscribed, fibro-calcified," and so on. A relatively recent analysis of lung lesions removed by surgery focused on two highly disparate structures, cavitary lesions and tuberculomas (Ulrichs and Kaufmann, 2006). Cavities were surrounded by few vessels and a diffuse pattern of proliferating cells, while tuberculomas exhibited a highly organized framework of follicle-like structures and high vascularization.

By describing variety and heterogeneity, pathologists have often tried to place different types of lesions in a seemingly logical temporal sequence to one another, insinuating developmental relationships (Dannenberg and Rook, 1994). Using sophisticated *in vivo* imaging reporter technology with molecular probes detecting host tissue metabolism as a measure for inflammatory activity, scientists are currently re-discovering this enormous heterogeneity of tuberculosis lesions, with the pioneers' advantage of following the same granuloma over time (Barry et al., 2009). Thus, "centroacinary, perifocal, tree-in-bud, metabolically active" have become useful phenotypic labels identifying several distinct types of lesions co-existing within a single individual. More surprisingly, these lesions may independently regress, and even vanish over time, while others flourish and exacerbate even under treatment (Barry et al., 2009; Lin and Flynn, 2010). There does not appear to be a clear linear relationship between these lesions, but rather a continuous spectrum. Macrophages within infiltrates form a scaffold to direct the movement of lymphocytes in search of antigenic stimulation (Egen et al., 2008, 2011), but both, a delay in T cell arrival as well as inhibition of T cell responses within the lesion account for a heterogeneous antimicrobial response and persistence of tubercle bacilli.

One theory contends that, even during latency, macrophages from time to time egress from the lesion and spread the infection to other parts of the lung (Cardona and Ruiz-Manzano, 2004; Cardona, 2009). Although this defies the very definition of a latent infection since migration of infective macrophages through the airspaces would effectively make this latent infection a patent one, it may reflect the flaring-up of quiescent lesions during apparent "latency." A consensus is emerging that not only active, but also latent disease shows a spectrum of lesional activity (Lin et al., 2009). In this respect, *in vivo* imaging may for the first time deliver functional classifications of diverse "latency" stages, superseding the current rather static description of lasting immune responsiveness to an infectious stimulus that occurred in the past (Mack et al., 2009). It would be interesting to apply the imaging approach to screen clinically healthy individuals in high-transmission populations for incipient active lesions. Therapeutic consequences are evident, in that the identification of functional biomarkers indicating latent infections would permit specific preventive chemotherapy of those individuals at greatest risk for reactivating tuberculosis (Russell et al., 2010). Interferon-α and neutrophil driven transcriptome signatures have recently been described as markers of susceptibility to active human tuberculosis (Berry et al., 2010). Experimentally, the absence of IFN-αβ signaling in mice improved outcome after infection with highly virulent but not with attenuated (Cooper et al., 2000; Manca et al., 2001), strains of *M. tuberculosis*, and genetically susceptible mouse strains survived longer when neutrophils were depleted (Keller et al., 2006). Whether these signatures can serve as prospective susceptibility markers for preventive treatment of latent tuberculosis patients has to be proven in longitudinal studies in high-transmission populations (Berry et al., 2010). One caveat is that some of these "biomarkers" may not be entirely specific for tuberculosis, but rather indicative of other chronic granulomatous conditions such as sarcoidosis (Maertzdorf et al., 2012).

A number of animal model systems, ranging from mice, guinea pigs, rabbits, minipigs, to marmoset, cynomolgous, and macaque non-human primates, are in use to depict various aspects of granuloma immunopathology. No single model system is perfect, but they all reflect individual aspects of human tuberculosis and, if taken with a grain of salt, contribute to stratifying the complexity of pathology in humans, which cannot yet be examined directly in molecular detail at the lesional site in humans. What has become

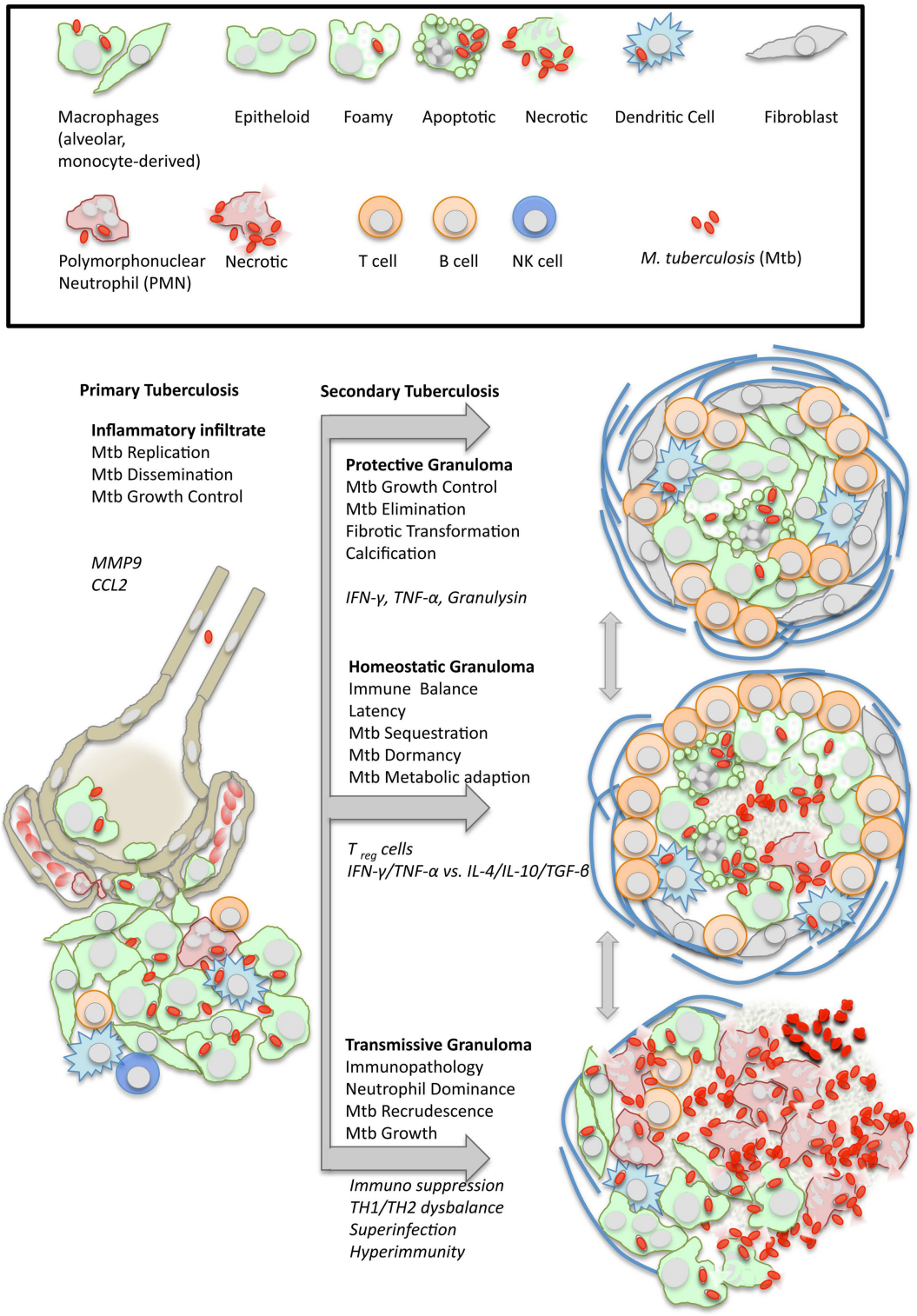

FIGURE 1 | Dynamics of granuloma formation and pathology in tuberculosis. *M. tuberculosis* (Mtb) elicits a local inflammatory infiltrate which may give rise to (i) protective immunity, (ii) balanced inflammation (i.e., control of Mtb growth with little tissue damage), or (iii) endobronchial transmission following granuloma necrosis. The depicted types of organized granulomas are idealized and represent stages of a pathophysiological continuum. At the same time, they represent stages of the Mtb life cycle with either retarded growth or metabolic adaptation within the granulomatous lesion, or recrudescence and spreading to the next host following granuloma disruption. Italics indicate typical cellular and humoral mediators involved in granuloma differentiation which are addressed in more detail in the text.

most evident from analyzing the diversity of granulomas is that no cross-sectional or even systemic marker can adequately represent the succinctly local microenvironments of pulmonary granulomas. Therefore, biomarker development is facing a formidable challenge!

VERSATILE METABOLISM WITHIN GRANULOMAS: NON-REPLICATING PERSISTENCE OF *M. TUBERCULOSIS*

Gluconeogenesis is necessary for *M. tuberculosis* throughout the infection, i.e., not only in the phase of persistence, and phosphoenolpyruvate kinase is the critical gate enzyme (Marrero et al., 2010). Within the granuloma, it is assumed that *M. tuberculosis* replicates only very little, but remains fully capable of generating energy (see **Figure 1**). Genome-wide expression profiling of *M. tuberculosis* RNA isolated from chronically infected mouse lungs or sputum of tuberculosis patients revealed transcriptional signatures reflective of environmental conditions such as low pH, oxygen depletion, iron limitation, nitrosative stress, and nutrient starvation (Timm et al., 2003; Voskuil et al., 2004; Talaat et al., 2007; Garton et al., 2008). This explains the attenuated phenotype of mutant *M. tuberculosis* defective in the leucin, lysine, purine, or pantothenate biosynthetic pathways, or which are deficient in iron acquisition or the stringent response (Boshoff and Barry, 2005).

The DosR regulon is a genetic program that critically governs survival in the absence of respiration (Leistikow et al., 2010). The conserved presence of long sequences of the DosR regulon in environmental mycobacteria suggests that it did not evolve specifically for survival within the mammalian host. Instead, the regulon may have evolved to cope with conditions within the environment such as very low oxygen tension. Thus, in a hypothetical ancestral *M. tuberculosis* strain, DosR gene products may have allowed the bacteria to survive in a specific niche in a non-replicating state, ensuring positive selection (Bartek et al., 2009).

Using a dual staining approach, three *M. tuberculosis* subpopulations were found in hypoxic culture and in lung sections of mice and guinea pigs. Bacteria were either exclusively acid-fast positive, exclusively immunofluorescent positive or acid-fast and immunofluorescent positive (Ryan et al., 2010). These results suggest that *M. tuberculosis* exists as multiple populations with distinct cell wall properties even within a seemingly single microenvironment, advocating the development of analytical tools at the single cell level. Indeed, intracellular mycobacteria have altered cell wall lipid pattern as those grown in broth (Fischer et al., 2001). Evidence from guinea pigs treated with antibiotics indicates that *M. tuberculosis* may persist extracellularly within biofilm-like structures that consist of DNA and neutrophil debris, in a hypoxic and iron-rich environment with incomplete dystrophic calcification (Lenaerts et al., 2007). These biofilms appear similar to those shown for biofilms of another lung pathogen, *Pseudomonas aeruginosa* (Parks et al., 2009). These niches where access of antibiotics is likely compromised may serve as the primary sites of disease reactivation, and mimicking these tissue conditions in axenic culture will be essential for successful *in vitro* compound screening for next generation anti-mycobacterials.

Even when the drugs reach the bacteria, the actual mechanism of killing *M. tuberculosis* remains unclear for most anti-tuberculosis drugs. A case in point is isoniazid. It is generally assumed that INH is only effective on actively replicating *M. tuberculosis*. The fact that INH preventive chemotherapy can reduce the level of tuberculosis manifestation in individuals diagnosed with latent infection has been a major argument for the contention that quiescent tuberculosis lesions contain actively dividing *M. tuberculosis* (Diel et al., 2008). It is clear that INH, following its activation by the catalase peroxidase KatG, inhibits mycolic acid synthesis. More specifically, an INH-NAD adduct inhibits the fatty acid synthase II enoyl-ACP reductase InhA, leading to the accumulation of long-chain fatty acids (Vilcheze and Jacobs, 2007). How exactly this precipitates *M. tuberculosis* death, however, is the subject of ongoing investigations at the single cell level within calibrated fermentation chambers, using fluorescent reporter probes (Golchin et al., 2012).

Detailing the mechanisms and kinetics of how drugs kill *M. tuberculosis in vitro* will be a crucial initial step for improving anti-tuberculosis drug efficacy. An even more challenging task will be to define and refine how drugs penetrate and work effectively within tuberculosis lesions. This requires full knowledge of the tissue microenvironment influenced by both, mycobacteria as well as host responses, and includes micronutrient availability for both "partners," defense cells and microbe. Innovative imaging techniques paired with fluorescent or luminescent reporter strains of *M. tuberculosis* are important tools for monitoring pathogenesis *in situ* and drug efficacy testing (Andreu et al., 2010; Carroll et al., 2010; Kong et al., 2010; Zelmer et al., 2012). Of even greater interest will be *M. tuberculosis* sensor strains, which can report physicochemical conditions *in situ* such as low iron concentration or drug exposure.

How does *M. tuberculosis* exit the state of seeming dormancy and resume growth? It may take one cue from the production of resuscitation promoting factors (Rpf), RpfB being the most relevant isoform out of five encoded in the *M. tuberculosis* genome based on persistence assays with targeted deletion mutants (Kana et al., 2008; see **Figure 1**). The structure of RpfB contains peculiar features that are also shared by G5 domains involved in biofilm formation (Ruggiero et al., 2009), providing a further link to evolutionarily conserved pathways of adaptation to adverse environmental conditions by changing growth patterns.

Foamy macrophages are key constituents of tuberculosis lesions, representing macrophages packed with lipid bodies following activation via Toll-like receptors and proinflammatory signals (D'Avila et al., 2006). These lipid bodies are the consequence of an imbalance between the influx and efflux of low-density lipoprotein particles from the serum. The phenotype can be induced *in vitro*, in peripheral blood cells incubated with distinct mycobacteria-derived oxygenated ketomycolic and hydroxylmycolic acids (Peyron et al., 2008). Lipid bodies may provide ideal nutrition for a bacterium that is changing its metabolism to digest fatty acids, as evidenced by upregulation of the gating enzyme of the glyoxylate shunt, isocitrate lyase, and of the methylcitrate pathway, methylcitrate dehydratase, in the intracellular life stage (McKinney et al., 2000; Gould et al., 2006; Mattow et al., 2006; Munoz-Elias et al., 2006).

Mycobacterium tuberculosis has also been shown to metabolize cholesterol (Pandey and Sassetti, 2008), and several genes such as Mce4, HsaC, and Icl1, which were previously linked

to propionate metabolism, may be functionally linked through cholesterol degradation. Cholesterol metabolism requires altering of transcription and metabolic profiles of *M. tuberculosis* to access propionyl-CoA and pyruvate pools through the methylcitrate cycle. Consequently, gene deletion mutants therein are handicapped for intracellular growth (Griffin et al., 2012). Analysis of lipids from the caseum of human tuberculosis granulomas revealed that the main lipid species are cholesterol, cholesteryl ester, triacylglycerides, and lactosylceramide, implicating low-density lipoprotein-derived lipids as the most likely source (Kim et al., 2010). Granuloma necrosis, at least in mouse *M. tuberculosis* or *M. avium* infection, is often associated with high bacterial burden, and virulent mycobacteria can inhibit membrane repair, which causes necrosis in infected macrophages (Divangahi et al., 2009). A conceivable scenario, therefore, is that the death of foamy macrophages results in the accumulation of lipid debris making up the caseum at the center of the granuloma (Russell et al., 2009; Behar et al., 2010). Other cells may also contribute to cellular debris as neutrophils represent a major cell population in TB lesions, which has an exquisitely high death toll due to their short half-life and *M. tuberculosis* driven necrosis (Eum et al., 2010; Corleis et al., 2012; see **Figure 1**).

The initial necrotic nidus may be induced in the absence of any adaptive immune response, as guinea pigs exhibit focal necrosis within granulomas as early as 10 days after infection (Turner et al., 2003). Overwhelming experimental and clinical evidence, however, suggests that promotion of full-fledged central caseation in mycobacterial granulomas requires a hypersensitivity reaction, precipitated by CD4+ T cells faced with a high antigenic load (Dannenberg, 1991; Ehlers et al., 2001; Sanghi et al., 2011).

PREPARING FOR THE EXIT: GRANULOMA NECROSIS AS A TRANSMISSION STRATEGY

There is much debate about whether tissue necrosis begins with central necrotization of preformed granulomas, or whether caseation and cavitation develop from so-called lipid pneumonia accompanying regrowth of *M. tuberculosis* persisting outside of granulomas (e.g., in epithelial cells, adipocytes, etc.; Hernandez-Pando et al., 2000; Neyrolles et al., 2006). Evidence from the former comes from studies in rabbits, guinea pigs, and designer mouse models, while evidence for the latter is derived from histopathological studies in men and mice. For example, mice bearing the sst-susceptible allele develop centrally necrotizing pulmonary granulomas in which *M. tuberculosis* growth is rampant. The host resistance gene, Ipr1, encoded within the sst1 locus, regulates the infected macrophages' mechanism of death (Kramnik, 2008). In dermally infected NOS2-deficient mice treated with a neutralizing anti-IFN-γ antibody, established granulomas centrally necrotize, showing signs of hypoxia and abundant cathepsin G activity (Reece et al., 2010). In guinea pigs, necrosis within granulomatous lesions can occur even before a robust T cell response has developed (Turner et al., 2003). In rabbits, cavitation has generally been attributed to a strong DTH response to *M. bovis* infection precipitating caseation, cavitation, and liquefaction of caseum within circumscript granulomas (Dannenberg and Sugimoto, 1976; Dannenberg and Collins, 2001).

By examining a histological archive of tuberculosis lesions in humans and revisiting the Cornell model of reactivation tuberculosis in mice, Hunter et al. (2007) came to the conclusion that reactivation of *M. tuberculosis* infection begins as a lipid pneumonia with bronchial obstruction and does not start from disintegrating granulomas. A very potent component of *M. tuberculosis* to which this necrosis-inducing activity is attributed is cord factor, or trehalose dimycolate which can induce inflammatory infiltrates including foam cell formation but is also a virulence factor promoting intracellular survival (Hunter et al., 2006; Kim et al., 2010). Taken together, caseation appears to correlate with pathogen-mediated dysregulation of host lipid metabolism.

As neutrophils represent the predominant cell population in broncho-alveolar lavages of active tuberculosis patients they may be more relevant in transmission of *M. tuberculosis* than in controlling infection (Eum et al., 2010). Considerable controversy exists over whether neutrophils are able to kill mycobacteria and a recent review on the issue concluded that these otherwise potent anti-bacterial effector cells fail to eliminate *M. tuberculosis* (Korbel et al., 2008). Indeed, virulent *M. tuberculosis* quickly cause necrotic cell death of human neutrophils upon infection *in vitro* by inducing reactive oxygen intermediates (ROI) allowing the mycobacteria to escape neutrophil-mediated killing. In contrast, an attenuated *M. tuberculosis* mutant lacking the virulence associated RD1 region fails to induce necrosis and falls victim to neutrophils' armamentarium, i.e., ROI (Corleis et al., 2012). More importantly, invading neutrophils with their payload of cytotoxic molecules may cause substantial tissue destruction and remodeling. It can be envisaged that misguided but anti-microbially ineffective neutrophils invade existing (latent?) tuberculosis lesions and prompt the immunopathological cascade toward active lesions, providing further host cell lipid substrate for *M. tuberculosis* growth and biofilm formation to secure subsequent transmission. Here, super-infection with a novel *M. tuberculosis* strain or even another unrelated co-infecting lung pathogen may represent an as yet under-appreciated cause of reactivation (see **Figure 1**). In addition, systemic co-infections of *M. tuberculosis* and unrelated pathogens beyond HIV may also modulate lung immunity in the latently infected. This was recently demonstrated in the murine model of malaria-associated acute respiratory distress syndrome, in which the mycobacterial burden was exacerbated in malaria – *M. tuberculosis* co-infected mice (Mueller et al., 2012).

Sequencing the genomes of 21 *M. tuberculosis* strains representative of the global diversity identified very little sequence variation in 491 experimentally confirmed human T cell epitopes, indicating purifying selection (Comas et al., 2010). This finding led to the hypothesis that *M. tuberculosis* benefits from recognition by human T cells. In view of its confinement to the granuloma, *M. tuberculosis* may have devised an exit strategy that exploits T cell activation. TH1 responses (IFN-γ, IRF-1) were shown to drive granuloma necrosis in a model of mycobacteria-induced pulmonary immunopathology (Ehlers et al., 2001; Aly et al., 2009), and TH2 responses have also been associated with increased cavity formation and tissue destruction in humans and mice (Rook, 2007); Hölscher, C., personal communication). Cavity formation in rabbits, monkeys, and man are clearly results of a long-lasting T cell-driven immune activation, low numbers

of CD4+ cells virtually precluding granuloma necrosis (Chaisson et al., 1987; Capuano et al., 2003). Moreover, AIDS patients with tuberculosis have a different pathology and often, their sputum is negative for acid-fast bacteria, further suggesting that transmission-facilitating granulomas are immune driven (Aaron et al., 2004, see **Figure 1**).

An integrated view of current findings would hold that individual components of *M. tuberculosis*, such as trehalose dimycolate, allow for foam cell formation and macrophage necrosis, but full-blown central necrosis of an established granuloma requires T cell participation, either in the form of a hyperactive IFN-γ production or a superimposed IL-4/IL-13 response. The latter induces alternative macrophage activation (Gordon and Martinez, 2010), and arginase 1 is a potential biomarker for reactivation tuberculosis and granulomas prone to necrotizing. Neutrophils, which are also potent producers of arginase 1, may even amplify this immunopathogenic cascade.

Are the "fat and lazy" persister bacilli present in the sputum of cavitary patients specifically adapted for transmission (Garton et al., 2008)? Very little is known about the physical parameters in individual *M. tuberculosis* bacteria relevant for aerosolization, survival in the environment, or infectivity (i.e., inoculum "take" in the alveolar macrophage; Fennelly et al., 2004). Also, there is a growing debate as to whether low dose aerosol infection adequately reflects the situation in endemic countries where exposure to putatively larger numbers of coughed-up *M. tuberculosis* may be the crucial factor overriding naturally existing or vaccine-induced immunity (Rook et al., 2009).

THE BOTTOM LINE: DOES *M. TUBERCULOSIS* UNIVERSALLY PROFIT FROM GRANULOMA FORMATION?

Given the recent focus on the stunning discovery that *M. tuberculosis* may direct granuloma induction and maturation as part of its life cycle, the fact that, in the absence of granuloma formation there is no protection, at least in the human host, has been unduly neglected. Granulomas afford a unique juxtaposition of activating T cells and mycobacteria-laden macrophages, and the temporal coincidence of T cell differentiation, granuloma consolidation, and reduction of *M. tuberculosis* growth in all animal models that mimic human disease suggests causality (North and Jung, 2004). It is important to emphasize that T cell memory affords 10-fold lower bacterial loads in infected organs of vaccinated animals, and that the majority of bacteria reside within granulomas and not randomly distributed throughout the body. Therefore, while a mycobacteria-focused view on granulomas was long overdue (Russell, 2007; Ramakrishnan, 2012), this should by no means neutralize the evidence in favor of the protective infection-sequestrating granuloma:

- T cells transfer protective immunity and granuloma formation (Orme and Collins, 1983).
- HIV-infected individuals exhibit poor granulomatous inflammation and poor control of mycobacterial growth (Lawn et al., 2002).
- Macrophage accumulations (or innate granulomas) do not efficiently kill mycobacteria unless activated by T cells (North and Izzo, 1993; Smith et al., 1997).
- Interferon-gamma provided by NK cells alone is insufficient to provide full protection in the absence of T cells (Feng et al., 2006).

It is certainly true that the host-centric view has prevailed for too long, neglecting the important input of *M. tuberculosis* in shaping the granuloma to its advantage, but it is unwise to underestimate the power of T cell-mediated macrophage activation, which takes place at the site of granulomatous inflammation, as it currently provides the only venue for preventive vaccination strategies.

CONCLUSION: IS IT POSSIBLE TO BE BETTER THAN NATURE?

One of the implications of the integrative view on *M. tuberculosis*' life cycle embraced here is that, to stop *M. tuberculosis* from multiplying and transmitting, simple imitation, or augmentation of the natural host response to infection is likely to fail. Unless T cells can be trained to recognize *M. tuberculosis* as soon as it enters the alveolar macrophage, one of the best vaccination strategies might be to bypass the regulatory networks *M. tuberculosis* itself initiates to establish its niche for replication. If anything, vaccines would have to mitigate TH1 and TH2 responses and altogether blunt regulatory T cell responses to allow more protective immunity while avoiding damaging pathology (Rook et al., 2005). This may be impossible to achieve purely by vaccination, leaving ample opportunity for adjunct immunomodulatory measures. Indeed, inhibition of inflammation may prove to be a superior adjunct strategy to improve the outcome of antibiotic therapy (Churchyard et al., 2009). For example, blockade of PDE4 together with INH shortened the duration of treatment by one month and reduced pathology in a rabbit model of tuberculosis (Subbian et al., 2011). During the Immune Reconstitution Inflammatory Syndrome (IRIS), which occurs in *M. tuberculosis*-infected AIDS patients receiving highly active antiretroviral therapy, corticosteroid therapy is highly effective. However, it does not reproducibly interfere with TH1 responses but reduces granzyme B and perforin levels, indicating an involvement of CD8+ T or NK cells in inflammatory exacerbation (Meintjes et al., 2009).

A potential problem with current immunization strategies against tuberculosis might be that they prime an immune response to epitopes that have been highly conserved in *M. tuberculosis*, because these dominant T cell targets ensure escape from the granuloma and thus transmission. Indeed, in an alternative strategy that refocused the T cell response to specificities that are not normally recognized during natural infection, a more efficient protection against murine *M. tuberculosis* infection was induced than afforded by immunization with the immunodominant epitope (Aagaard et al., 2009). This increased efficacy was associated with elevated numbers of multifunctional T cells, producing TNF, IFN-γ, and IL-2 at high levels.

Further considerations for improving vaccines take into account that *M. tuberculosis* responds to the host immune response by regulating and diversifying its own gene expression; for example, during latency, stage-specific antigens are expressed that represent its metabolic adaptation and can effectively be utilized to discriminate, by immunodiagnosis of host T cell responses against

these antigens, between acute and latent infection (Demissie et al., 2006). Based on these findings, a novel subunit combination vaccine was developed: H56, comprising three *M. tuberculosis* antigens, which are expressed either early in infection (Ag85, ESAT6) or during the latent phase (Rv2660c), not only boosts BCG-primed immunity but also induces multifunctional T cell-mediated immune protection on its own before and after exposure to *M. tuberculosis* (Aagaard et al., 2011). Ag85 is expressed by both, BCG and *M. tuberculosis*, and is therefore responsible for the boost effect, whereas ESAT6 is exclusive for the latter one and expressed mostly during early, active replication stages. Rv2660c however, is expressed during the entire course of infection in mice (even by starved and dormant *M. tuberculosis*) but is only a weak IFN-γ inducer by itself. It becomes mildly immunogenic only when fused to Ag85-ESAT6 thereby obviating immune-mediated exacerbation of the disease in infected individuals. In sum, the selection and combination of antigens and epitopes specific for different stages of TB may help outwit *M. tuberculosis* and control reactivation.

It would be unreasonable to call the TB granuloma an unsuccessful host defence, as it successfully contains the infectious focus in more than 90% of cases. The 10% of individuals that progress toward TB disease suffer from a disbalanced inflammatory reaction, be it due to too little innate or adaptive immunity or due to unrestrained hypersensitivity reactions. There is no balance without a trade-off: in the case of TB this is the Janus face of T cell immunity (which can be detrimental when overzealous). Any intervention thus must be regulatory in nature rather than proinflammatory, and the rational design of therapies and vaccines must take this into account.

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Within the Enemy's Camp: contribution of the granuloma to the dissemination, persistence and transmission of *Mycobacterium tuberculosis*

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Pulmonary tuberculosis, caused by *Mycobacterium tuberculosis (M.tb)* represents a leading global health concern, with 8.7 million newly emerging cases, and 1.4 million reported deaths annually. Despite an estimated one third of the world's population being infected, relatively few infected individuals ever develop active clinical disease. The ability of the host to remain latently infected while preventing disease is thought to be due to the generation of a robust type 1 immune response in the lung, capable of controlling, but not clearing, *M.tb*. A key feature of the type 1 immune response to *M.tb* is the formation of immune cellular aggregates termed granuloma. The granuloma structure has long been considered a hallmark of host's protective response toward *M.tb*. Historically, a correlative relationship between granuloma formation/maintenance and bacterial control has been seen in models where disrupted granuloma formation or structure was found to be fatal. Despite this established relationship much about the granuloma's role in *M.tb* immunity remains unknown. Recent publications suggest that the granuloma actually aids the persistence of *M.tb* and that the development of a necrotic granuloma is essential to person-to-person transmission. Our group and others have recently demonstrated that enclosed within the granuloma is a population of immunologically altered antigen-presenting cells and T lymphocyte populations. Of note, the ability of these populations to produce type 1 cytokines such as interferon-gamma, and bactericidal products including nitric oxide, are significantly reduced, while remaining competent to produce high levels immunosuppressive interleukin-10. These observations indicate that although the chronic granuloma represents a highly unique environment, it is more similar to that of a tumor than an active site of bacterial control. In this review we will explore what is known about this unique environment and its contribution to the persistence of *M.tb*.

Keywords: tuberculosis, immunopathology, immune regulation, granuloma, bacterial persistence, mycobacteria

INTRODUCTION

Pulmonary tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis (M.tb)* is the leading cause of death due to a bacterial pathogen and is responsible for 1.4 million deaths annually, latently infecting one third of the world's population (WHO, 2012). Despite the magnitude of individuals infected, the rate of mortality is relatively low with approximately 90% infected individuals controlling, but not clearing, *M.tb* (WHO, 2012). The ability of the host to "control" *M.tb* infection encompasses a number of immunological processes designed to restrain bacterial dissemination and persistence, and reduce person-to-person transmission. The classical hallmark of anti-TB host defense is the formation of type 1 immune granuloma in the lung. Historically, the granuloma has been perceived as essential to anti-TB host defense as the host is incapable of sterile clearance and thus is forced to segregate the infected cells as a means to preserve itself. However, experimentally little is known of the role of the granuloma in bacterial control.

THE HISTORICAL VIEW OF MYCOBACTERIAL GRANULOMA

First described in 1679, pathologists discovered unique structures in the lungs of TB patients (de le Boe, 1679; reviewed in Ramakrishnan, 2012). These structures were then termed tubercles and represent what we now know as granulomas. Commonly, it was observed that persons who had died of TB had a large number of these distinct pathological lesions, and the presence of tubercles became an associated hallmark of active TB disease. It was not until 1884 that tubercles were also characterized in individuals who had died from diseases other than TB. Upon post-mortem examination, a number of these individuals had lung lesions (granulomas) containing live TB bacilli, giving the first indication that TB latency may relate to the formation of granuloma (Dejerine, 1884). However, upon further microbiological examination, it was revealed that live bacilli persisted not only within the granuloma itself, but also in the surrounding lung tissue, albeit to a lesser degree (Wang, 1916; Opie and Aronson, 1927; Robertson, 1933; Feldman and Baggenstoss, 1938, 1939). It was around this time that the

protective view of the granuloma began to gain public acceptance and it was proposed that the recruitment of activated lymphocytes and the formation of a lymphocytic cuff served to wall-off infected macrophages as a means of limiting dissemination. However, the role of granuloma in TB has remained enigmatic largely because of the unavailability of reliable animal models and appropriate techniques to observe the dynamic process of granuloma evolution. Although different experimental models (mice, guinea pig, rabbit, cattle, and macaque) have been developed, only cattle and macaque monkeys form the type of granuloma that closely resembles those seen in humans (Capuano et al., 2003; Flynn et al., 2003; Tsai et al., 2006; Hunter et al., 2007; Via et al., 2008). Despite being extensively used as a model of TB, the murine granuloma lacks many of the unique characteristic features of the human granuloma including centralized necrosis, giant multinuclear cells, and a defined "lymphocytic cuff" (Rhoades et al., 1997). Contrasting the classical notion of its protective role, a number of recent studies have demonstrated unaltered bacterial control even in the absence of granuloma formation, strongly arguing against the granuloma being essential to bacterial restriction (Johnson et al., 1998; Scott and Flynn, 2002; Pearl et al., 2004). Moreover, it is now known that the *Mycobacterium* can significantly alter the immune environment of the granuloma as means to facilitate its persistence (Ly et al., 2007; Scott-Browne et al., 2007; Marino et al., 2010; Castano et al., 2011; O'leary et al., 2011). Regardless, the common perception remains that the granuloma serves to limit bacterial growth and prevent dissemination by segregating infected cells, and the role of the granuloma in *M.tb* infection remains an issue of continued debate. In this review we will challenge the traditional view of the function of granuloma, exploring what is known about its progression and maturation, and how this unique environment may in fact contribute to the persistence and transmission of *M.tb.*

FORMATION OF THE TYPE 1 IMMUNE GRANULOMA DURING *M.tb* INFECTION

The formation of granuloma is a dynamic process that begins shortly after infection and continuously evolves over time. Temporally, the granuloma can be divided into three distinct phases: (1) the "innate granuloma," a loose aggregate composed primarily of recruited macrophages and neutrophils; (2) the "immune granuloma" formed following the emergence of antigen-specific T cells; and (3) the "chronic granuloma," resulting from distinct morphological changes in granuloma structure (**Figure 1**).

FORMATION OF THE "INNATE GRANULOMA"

Shortly after aerosol exposure, *M.tb* infects the resident alveolar macrophage (AM) initiating the early inflammatory response. While amplifying the host immune response, the recruitment of innate immune cells inadvertently provides a large number of new targets for *M.tb* to infect and is thought to contribute to the early dissemination of *M.tb* (Doenhoff, 1997; Davis and Ramakrishnan, 2009). Augmenting this problem, the infected AM is unable to kill internalized mycobacteria due to impaired phagolysosome fusion, a process essential to the destruction of the phagocytosed bacteria (Welin et al., 2011). The efficiency by which a *Mycobacterium* species arrests phagolysosome fusion is directly attributable to its relative virulence, with highly virulent strains such as *M.tb* almost completely inhibiting fusion (Ferrer et al., 2010). Incapable of killing internalized *M.tb*, infected macrophages secrete an array of pro-inflammatory and chemoattractant cytokines including tumor necrosis factor (TNF), interleukin (IL)-6, and IL-8 which facilitate the recruitment of new macrophages and granulocytes to the site of infection and lead to the formation of the "innate granuloma" (Birkness et al., 2007). This initial recruitment is essential to establishing the macrophage-dominated center of the "immune" granuloma.

CONTRIBUTION OF *M.tb* TO THE FORMATION OF THE "INNATE GRANULOMA"

Historically the formation of granuloma has been considered to be a host-mediated event. Using an *M. marinum* model, real-time microscopic visualization has challenged this notion, revealing that virulent *Mycobacterium* drives the nascent formation of the early granuloma. A number of elegant studies conducted by Ramakrishnan's group have demonstrated the unique interplay between the *Mycobacterium* and the host immune system in the early stage of granuloma formation. To this end, the early release of 6 kDa early secretory antigenic target (ESAT-6) by *M. marinum* led to the activation of the epithelium, which facilitated the recruitment of macrophages to the site of infection through inducing the production of matrix metalloproteinase-9 (MMP-9) (Davis and Ramakrishnan, 2009; Volkman et al., 2010). In murine models, *M.tb* was also found to drive MMP-9 expression. Thus either broad MMP inhibition or MMP-9-specific depletion delayed granuloma formation, resulting in impaired macrophage recruitment to the site of infection and reduced granuloma size (Taylor et al., 2006). These findings indicate that *M.tb* may actually promote granuloma formation and utilizes the structure for its own benefit. Furthermore, it was demonstrated that virulent mycobacteria can utilize the innate granuloma as means of recruiting target cells allowing for the early dissemination of mycobacteria throughout the host (Davis and Ramakrishnan, 2009). This view is supported further by the observation that the early granuloma is not a static environment and there is a significant movement of antigen-presenting cell (APC) populations into and out of the early granuloma (Chiu et al., 2004; Egen et al., 2008, 2011; Schreiber et al., 2011).

FORMATION OF THE "IMMUNE GRANULOMA"

Following innate activation, dendritic cells are recruited to the lung and transport mycobacteria or mycobacterial antigens to the mediastinal lymph node (MLN). Within the MLN antigen-loaded APCs activate antigen-specific T cells. Due to the nature of *M.tb* infection, the majority of bacilli and antigen reside within an endosome, and are most efficiently loaded onto major histocompatibility complex (MHC) class II (Mogues et al., 2001; Anis et al., 2008; Yahagi et al., 2010). The loading of MHC class II facilitates the priming of Th1 interferon-gamma (IFN-γ)-secreting T cells, which rapidly home to the lung. While the dominant subset of T cells are $CD4^+$, cross-presentation also allows for the strong induction of $CD8^+$ T cells, collectively generating a type 1 polarized adaptive immune response (Winau et al., 2006). Although outside the scope of this review, it should be noted that there is a substantial lag period between *M.tb* infection and the initiation of antigen-specific T cell responses (reviewed in Shaler et al., 2012).

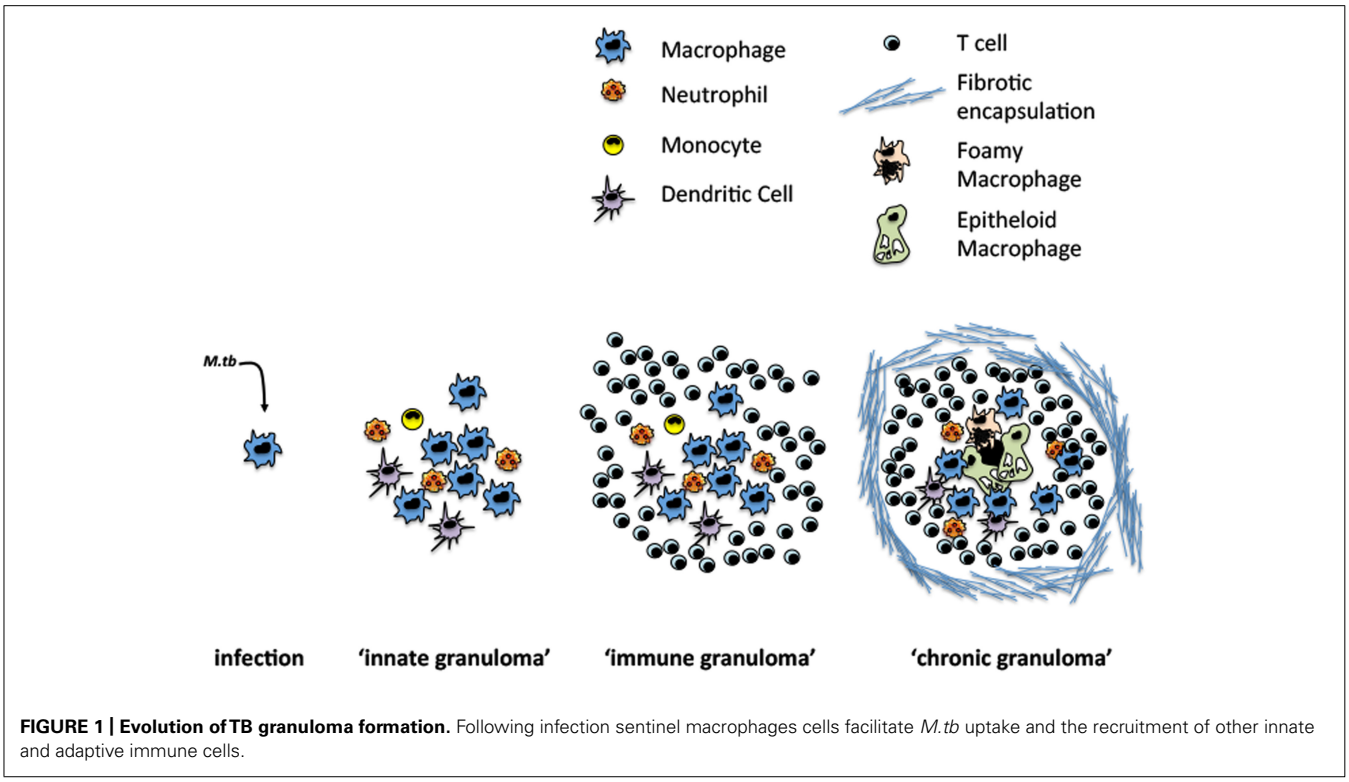

FIGURE 1 | Evolution of TB granuloma formation. Following infection sentinel macrophages cells facilitate *M.tb* uptake and the recruitment of other innate and adaptive immune cells.

The continuous production of chemokines by infected lung APCs efficiently recruits newly primed T cells into the lung. Once in the lung, recruited T cells surround and wall-off infected macrophages, activate them for enhanced bactericidal function, and physically limit their mobility to restrain bacterial dissemination. Indeed, the arrival of effector T cells and the establishment of the classical "immune granuloma" is associated with a plateau in bacterial growth (Mogues et al., 2001). While the prevailing immune response generated following *M.tb* infection is highly similar between the mouse and man, the structural formation of the "immune granuloma" differs significantly. In mice, many of the hallmark features of the human granuloma are missing, and thus the knowledge from murine granuloma research should be interpreted with caution. Despite these limitations, animal models have provided significant insight, and have been invaluable in delineating the stages of granuloma formation.

THE CONTROVERSIAL REQUIREMENT OF THE "IMMUNE GRANULOMA" IN LIMITING BACTERIAL DISSEMINATION

In spite of the traditional protective view of granuloma, it has recently been revealed that the immune environment within granuloma is more conducive to *M.tb* persistence than its elimination. Despite the fact that animal models do not accurately replicate human granuloma structures, several murine studies have provided invaluable insight into the role of granuloma in preventing *M.tb* dissemination control. Early studies demonstrating the role of critical cytokines such as TNF and IFN-γ perpetuated the notion that the granuloma was essential for bacterial segregation and limiting bacterial growth in the lung as the absence of either cytokine led to ill-formed granuloma and increased bacterial infection (Flynn et al., 1998; Kaneko et al., 1999; Algood et al., 2005; Beham et al., 2011; Gallegos et al., 2011). Moreover, any loss of $CD4^+$ T cell functionality results in a loss of the granuloma structure and extensive bacterial dissemination is seen in both man and mouse. While essential to the control of *M.tb*, the role of the $CD4^+$ T cell in the granuloma's structure is somewhat species-dependent. Specifically, in humans, T cells surround and wall-off infected macrophages, and do not infiltrate the granuloma, but rather form a defined lymphocytic cuff. Conversely, murine $CD4^+$ T cells associate directly with infected cells infiltrating throughout the granuloma forming lymphocytic aggregates or pseudo-granulomas (Mogues et al., 2001; Tsai et al., 2006; Hunter et al., 2007; Via et al., 2008; Gallegos et al., 2011; Geldmacher et al., 2012). Nevertheless, while the loss of CD4 T cell-mediated immunity is detrimental to the host it is impossible to separate the relative contribution of the two processes to the impaired bacterial control: loss of Th1-mediated immunity and loss of granuloma structure, these studies suggest a critical role for the granuloma in preventing bacterial dissemination (Saunders et al., 2002; Segovia-Juarez et al., 2004). The essential role of CD4 T cells to the control of mycobacterial growth has largely been attributed to their potent IFN-γ production and subsequent macrophage activation. While macrophage activation is essential to the control of *M.tb* in mouse and man, the role of nitric oxide has been an issue of some debate. Historically, studies have shown that human macrophages do not produce nitric oxide to the same degree *in vitro* as those isolated from mice (Aston et al., 1998). Recently, however, several groups have demonstrated that human macrophages from TB infected patients, as well as human macrophage cells lines are capable of inducing iNOS and producing nitric oxide in response

to *M.tb* antigen (Rich et al., 1997; Jagannath et al., 1998; Dlugovitzky et al., 2000). Indeed, it appears that while the timing of nitric oxide and its role in TB control may differ somewhat between mouse and man, nitric oxide remains important in both species.

Recent studies suggest that the granuloma may be dispensable for preventing bacterial dissemination and may actually contribute to *M.tb* persistence. Moreover, in the absence of intracellular adhesion molecule-1 (ICAM-1), there is also a failure of granuloma formation, and despite this defect, mice are protected for the first 90 days post-infection, with no increase in bacterial growth compared to wildtype mice within this time frame (Johnson et al., 1998). Similarly, zebrafish models have shown that in the absence of early granuloma formation, there is no defect in the ability of the host to limit bacterial replication and dissemination, and that the granuloma may actually facilitate early dissemination (Volkman et al., 2004, 2010). Furthermore, in the absence of IL-27 in mice, there is a substantial defect in the ability of the host to form granuloma in response to *M.tb* infection, and yet infected mice exhibit markedly enhanced bacterial control when compared to their wildtype counterparts (Pearl et al., 2004). Indeed, recent studies indicate that granuloma does not always function to limit bacterial dissemination. For example, C-C chemokine receptor type 2 (CCR2) deficient mice form exaggerated granuloma structures when infected with *M.tb* and paradoxically have a decreased capacity to control bacterial growth (Scott and Flynn, 2002).

While it remains debatable whether the granuloma is required for bacterial control, growing evidence supports the notion that the fate of *M.tb* within the granuloma is situation-dependent. For example, the initial inoculum size may influence the number of macrophages and granulocytes that are recruited to the site of infection. If a large number of cells are initially recruited, spatially it becomes difficult for effector T cells to interact with the infected cells residing at the core of the granuloma limiting their ability to activate these centralized macrophages to kill internalized *M.tb* (Segovia-Juarez et al., 2004). In comparison, a small initial inoculum size infects a small number of cells at the core of granuloma, which may increase the likelihood of interaction of infected cells with effector T cells (Segovia-Juarez et al., 2004). Based on this notion, it is tempting to speculate that a stronger initial innate immune response may perpetuate the infection and limit the host's ability to eliminate *M.tb*. Therefore, a small-size granuloma may favor host defense whereas a relatively large-size counterpart may favor the persistence of mycobacterial bacilli, regardless of the magnitude of T cells generated. The current limitation to diagnostic imaging makes studying the evolution of the granuloma in latently infected humans difficult and as a consequence, little is known about how its structure changes over the course of infection in otherwise healthy individuals.

THE "IMMUNE GRANULOMA": A NICHE FOR BACTERIAL PERSISTENCE

Regardless of whether the granuloma functions to limit bacterial dissemination, much evidence suggests that *M.tb* is especially adept at altering the immune response within the granuloma, creating a uniquely suppressed environment largely through the induction of IL-10 (de Waal Malefyt et al., 1993; Chiu et al., 2007, 2008; Higgins et al., 2009; Marino et al., 2010; Redford et al., 2010; O'leary et al., 2011; Shaler et al., 2011). Functionally, the infected macrophages within the granuloma are altered, showing a reduced capacity to produce bactericidal products such as nitric oxide, while showing enhanced IL-10 production (de Waal Malefyt et al., 1993; Chiu et al., 2007, 2008; Higgins et al., 2009; Marino et al., 2010; Redford et al., 2010; O'leary et al., 2011; Shaler et al., 2011). Interestingly, while the macrophage populations of the granuloma have reduced bactericidal function, they continue to produce large amounts of chemokines facilitating the continuous recruitment APC populations into the granuloma (Schreiber et al., 2010; Shaler et al., 2011). Recent studies utilizing *intravital* microscopy have revealed significant movement of inflammatory APCs both into and out of the granuloma (Schreiber et al., 2011). It is this movement of infected APCs that has been speculated to facilitate the early dissemination of *M.tb*. Likewise, human granuloma contains a high frequency of foxp3+ T regulatory cells (Rahman, 2009). In addition, murine studies have confirmed that T cells residing within the granuloma display a highly altered, and functionally suppressed phenotype. Despite the central role of IL-10 in suppressing T cell and macrophage activation within the granuloma, IL-10 neutralization or infection of IL-10 knockout (KO) mice results in only marginally reduced bacterial loads (de Waal Malefyt et al., 1993; Jacobs et al., 2002; Chiu et al., 2007; Higgins et al., 2009). Given *M.tb's* long evolution with humans, it is not surprising that *M.tb* targets multiple pathways to interrupt the host immune response. Moreover, while conventionally immune suppression would appear to benefit only the pathogen, the induction of IL-10 may actually be a host-mediated event required to limit unwanted immunopathology.

THE "CHRONIC GRANULOMA": A DYNAMIC INTERPLAY BETWEEN PERSISTING *M.tb* AND THE HOST IMMUNE RESPONSE

Following the establishment of the "immune granuloma," a period of immune quiescence is established. It is during this stage that chronic immune activation leads to significant alterations in the morphology and functionality of the granuloma, many of which are not typically seen in mice. Post-mortem studies of latently infected humans and non-human primates have revealed that the evolution of the granuloma structure is highly divergent, not only between individuals, but even within a single individual (Capuano et al., 2003; Flynn et al., 2003; Barry et al., 2009). Typically, within an infected individual, a spectrum of granuloma structures are seen. For instance, both the fully calcified lesions containing no bacilli and the fibrotically encapsulated necrotic granulomas containing large numbers of live bacteria can be seen in the lung of the same individual, indicating that the evolution of the granuloma is a highly dynamic process (Capuano et al., 2003; Flynn et al., 2003; Barry et al., 2009).

While the granuloma has long been believed to be a protective host's response, it is now acknowledged to result from a dynamic and continuous interplay between the host's immune response and persisting *M.tb*. The continuous "battle" between chronic immune activation and bacterial persistence causes infected macrophages to adopt an irregular epithelial-like or "epithelioid" appearance, and to fuse together forming multinucleated giant cells in the core of granuloma (Hunter et al., 2007; Hunter, 2011). Indeed, the virulent *Mycobacterium* has been shown to induce macrophage cell

death frequently throughout the course of infection, which several studies have shown to be a potential mechanism of gaining access to new host macrophages (Davis and Ramakrishnan, 2009). Newly recruited macrophages quickly phagocytose the dead bodies and become saturated with mycobacteria and lipids (Peyron et al., 2008). These lipid-rich macrophages that accumulate within the granuloma are known as foamy macrophages due to their distinct appearance, and are now recognized as a major contributing factor in the persistence of *M.tb* (Peyron et al., 2008). Recent studies have shown that foamy macrophages isolated from humans have lost key functions including their ability to phagocytose and to produce essential bactericidal agents such as nitric oxide (Peyron et al., 2008). Moreover, the polarization of macrophage populations within the granuloma are thought to shift from being a classically activated (M1) population toward that of an alternatively activated (M2), with reduced bactericidal capacity (Redente et al., 2010). Thus, such foamy macrophages have been proposed to function as the reservoirs of *M.tb* whereby the bacterium is able to successfully manipulate the infected macrophage into not only a safe haven but also a source of nutrients required for the synthesis of its cell wall and replication.An infected host typically houses a highly heterogeneous mixture of granuloma types ranging from large necrotic granulomas containing large amounts of bacilli, to completely calcified structures devoid of any detectible bacteria (Turner et al., 2003; Hunter et al., 2007; Hunter, 2011). In the later stages of granuloma evolution, fibrotic encapsulation can be seen in cases of both active and latent infection. Currently, it is unclear whether encapsulation functions to prevent bacterial escape, or to limit immune infiltration into the granuloma. Although complete mycobacterial clearance is rarely seen, latently infected humans display the evidence of healed granulomas, marked by central calcification in conjunction with fibrotic encapsulation containing no detectible bacilli (Opie, 1917). Despite the observed absence of bacteria within highly calcified granulomas, it is currently unclear whether this represents immune-mediated clearance, or simply a structural artifact left behind following *M.tb* escape.

Mycobacterium tuberculosis FACILITATES PERSON-TO-PERSON TRANSMISSION THROUGH ALTERATIONS TO THE GRANULOMA

Despite the attempts of the host to contain *M.tb* within the granuloma, as the infection progresses, the majority of individuals will develop granulomas with a necrotic focus formed due to the caesation of the macrophage infused center (Kim et al., 2010; **Figure 2**). This eventual necrosis of the granuloma is now accepted as a necessary event in facilitating the transmission of *M.tb* by disrupting the lung structure and allowing *M.tb* to gain access to the major airways. It should be noted that, in addition to person-to-person transmission, the active granuloma leaking bacteria into the airways may also allow for intrapulmonary dissemination (Cardona, 2009, 2010; Cardona and Ivanyi, 2011). It is therefore likely that the heterogeneity in granuloma structure seen in different lung regions of the same host represents different evolutionary timelines. Although much remains to be understood, it is clear that the evolution of granuloma is the result of the dynamic interplay between persisting mycobacteria and the host immune response, continuously evolving throughout the course of *M.tb* infection. Interestingly, *M.tb* may actually utilize the host

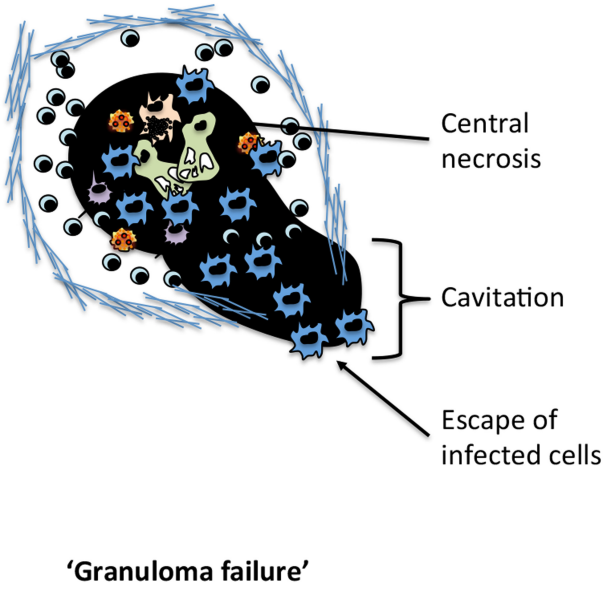

FIGURE 2 | The failure of the TB granuloma. The characteristic changes associated with the disintegration of the TB granuloma required for dissemination.

immune response to facilitate the structural changes required to facilitate person-to-person transmission. In line with this, while essential to preventing bacterial dissemination, paradoxically IFN-γ-producing Th1 cells may also play an integrate role in facilitating bacterial transmission (Ehlers et al., 2001). The ability of *M.tb* to manipulate the host immune response as means to facilitate central granuloma necrosis and facilitate its transmission while deterring immune-mediated bacterial clearance is a remarkable but poorly understood feature of *M.tb*. It is important to note that the processes of bacterial dissemination within a host, and transmission between hosts may be independently regulated. For instance, clinically it has been observed that despite exaggerated bacterial burdens and extensive dissemination, HIV-AIDS individuals co-infected with *M.tb* transmit *M.tb* person-to-person far less efficiently (Doenhoff, 1997; Ledru et al., 1999; Corbett et al., 2003; Glynn et al., 2008). The inability of the HIV-infected host to spread *M.tb* has been attributed to a failure of *M.tb* to drive central granuloma necrosis and cavitation, and the transport of bacilli to the airway. These observations argue that *M.tb* utilizes the necrotic granuloma as a portal for person-to-person transmission.

While it is well-known that changes to the granuloma structure are required for bacterial transmission, it is currently unknown whether the host or bacteria are responsible for these changes. Recently, studies have examined the granuloma at early and chronic stages of disease revealing a dramatic shift in the genes expressed by both *M.tb* and the host immune response. Notably, within the granuloma, the host immune response shifts from predominately pro-inflammatory during the early phases of infection, to immunosuppressive during the chronic stages (Karakousis et al., 2004; Ly et al., 2007; Mehra et al., 2010). Coincidentally, *M.tb* expresses a defined set of genes that function to facilitate immune

activation, while simultaneously expressing enzymes to combat immune-mediated clearance (Rohde et al., 2012). This is later followed by a shift in gene expression thought to facilitate immune senescence within the granuloma, allowing for *M.tb's* persistence (Rohde et al., 2012). While traditionally *M.tb* is thought to lie dominant, it has recently been demonstrated that throughout the course infection *M.tb* will periodically "awaken," up-regulating a number genes and sample the immune environment (Karakousis et al., 2004). This sampling allows *M.tb* to identify the optimal conditions for facilitating person-to-person transmission. Moreover, during the caseous stage of granuloma formation there is a further shift in the genes expressed by *M.tb* with a significant up-regulation of genes associated with lipid metabolism (Kim et al., 2010). Notably, most mycobacterial species are rich in immunomodulatory lipids, which play a central role in immune evasion. Intriguingly, the distribution and release of certain lipids by *M.tb* varies significantly over the course of infection, providing a means by which *M.tb* directs the host immune response. To this end, *M.tb* can release toxic lipids and generate targeted tissue damage. The generation of central necrosis is essential to facilitating cavitation and promoting *M.tb* transmission. Trehalose 6,6′-dimycolate (cord factor) has potent cytotoxic effects and has been implicated in the generation of central necrosis of the granuloma and the transmission of *M.tb* (Hunter et al., 2006; Kim et al., 2010). Recently, it has been shown that neutrophils and AMs recognize mycobacterial cord factor through their surface c-type lectin receptor, mincle (Behler et al., 2012; Lee et al., 2012). The engagement of mincle leads to a pro-inflammatory cytokine pathway that aids in the early cellular recruitment and control of mycobacteria (Behler et al., 2012; Lee et al., 2012). Interestingly, however, the proportion of cord factor varies greatly throughout the course of infection with its synthesis heavily up-regulated by *M.tb* during the development of central necrosis and cavitation (Hunter et al., 2006; Kim et al., 2010). Indeed, studies have linked the amount of cord factor released by *M.tb* to the extent of necrosis and cavity formation (Hunter et al., 2006; Kim et al., 2010). Moreover, previous studies have documented mincle as a key receptor in the detection of necrosis and the development of an inflammatory response upon tissue damage (Yamasaki et al., 2008). Given that cord factor is known for its cytotoxic effects, one may speculated that engaging mincle may be central to the development of a pro-inflammatory response capable of aiding the formation of cavitation within the granuloma. Utilizing the host immune machinery *M.tb* facilitates the necessary structural changes to ensure its own transmission, which occurs at a time when the immune system is most vulnerable.

CONCLUDING REMARKS

While the true nature of the granuloma still remains to be defined, it is now clearly evident that the granuloma is not just a host-mediated entity of segregation and rather, it is a dynamic battlefield bearing the scars left both by the pathogen and the host immune response. While it may have been originally destined to restrain bacterial dissemination, *M.tb* efficiently hijacks the granuloma to provoke the generation of an immunologically sheltered niche to reside within and persist until the situation is favorable to bacterial transmission.

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Emerging trends in the formation and function of tuberculosis granulomas

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The granuloma is an elaborated aggregate of immune cells found in non-infectious as well as infectious diseases. It is a hallmark of tuberculosis (TB). Predominantly thought as a host-driven strategy to constrain the bacilli and prevent dissemination, recent discoveries indicate the granuloma can also be modulated into an efficient tool to promote microbial pathogenesis. The aim of future studies will certainly focus on better characterization of the mechanisms driving the modulation of the granuloma functions. Here, we provide unique perspectives from both the innate and adaptive immune system in the formation and the role of the TB granuloma. As macrophages (Mϕs) comprise the bulk of granulomas, we highlight the emerging concept of Mϕ polarization and its potential impact in the microbicide response, and other activities, that may ultimately shape the fate of granulomas. Alternatively, we shed light on the ability of B-cells to influence inflammatory status within the granuloma.

Keywords: macrophage, B-cells, mycobacteria, tuberculosis, granuloma

INTRODUCTION

> *"On the basis of my numerous observations I consider it established that, in all tuberculous affections of man and animals, there occur constantly those bacilli which I have designated tubercle bacilli and which are distinguishable from all other microorganisms by characteristic properties."*

With those celebrated words in 1882, Koch announced the discovery of the etiological agent of one of the oldest recorded human afflictions (Koch, 1982). The term "tubercle" refers to an original description by Sylvius (in 1650) of the apparent lung nodules characteristic of the "consumption" disease, which became christened as "tuberculosis (TB)" by Schonlein (in 1839) in recognition of its intricate correlation with these structures (Sakula, 1982). Today, these tubercles are known as granulomas, defined as organized immune cell aggregates that form in response to persistent TB infection (Ramakrishnan, 2012). The cellular composition of TB granulomas includes Mϕs, neutrophils, monocytes, dendritic cells, B- and T-cells, fibroblasts, and epithelial cells (Russell, 2007; Ramakrishnan, 2012). Moreover, TB granulomas are characterized by a high-turnover rate of their Mϕ population and by specialized differentiations taking place in mature Mϕs such as tightly interdigitated cell membranes that make Mϕs appear either epithelial (Adams, 1974), fusion into multinucleated giant cells (Helming and Gordon, 2007), or differentiation into foamy cells with a high lipid content (Russell et al., 2009). While granulomas have been studied for about 200 years, their role in TB etiology remains unclear. In 1819, Laënnec first proposed granulomas as the cause of TB (Sakula, 1982). Yet, about a century went by before Ghon correlated the presence of a single caseous granuloma in the mid-region of the lung with a corresponding nodal involvement (the Ghon complex) and the pathogen's dissemination, thus serving as a marker for latent TB (Dorhoi et al., 2011). In spite of this, subsequent studies and clinical observations established the granuloma as a host-protective structure that "walls off" Mtb to prevent its dissemination, a notion that still predominates. Seminal studies by Ramakrishnan in zebrafish, however, have now evidenced mycobacteria actually exploit the granuloma into a tool for pathogenesis, suggesting its function can be modulated depending on the disease context (Ramakrishnan, 2012). Considering TB is still one of the leading causes of human death due to a single infectious agent, substantial insights into microbe physiology and host defenses rest in the attempt to better understand the mechanisms governing TB granulomas.

Here, we will focus exclusively in the role of Mϕ polarization in the formation and function of TB granulomas. Likewise, we will provide a unique perspective on the significance of B-cells, whose immune-modulatory function has long been ignored in TB.

MACROPHAGE POLARIZATION IN TB GRANULOMAS

Mϕ polarization is broadly classified into M1 and M2 programs (Goerdt and Orfanos, 1999; Gordon, 2003; Mantovani et al., 2004; Martinez et al., 2009). On one hand, the M1 program is a response to type-1 inflammatory conditions (e.g., IFN-γ), often associated with intracellular pathogen resistance (Quintana-Murci et al.,

2007; Benoit et al., 2008). IFN-γ is mainly responsible for the establishment of the M1 program, granting Mϕs the capacity to kill mycobacteria (Flynn et al., 1993; Ehrt et al., 2001). The production of nitric oxide (NO) in Mϕs (characteristic in murine models) is arguably one of the most important consequences mediated by IFN-γ, as mice deficient for NO production succumb to Mtb infection (Chan et al., 1992). In fact, the enzyme iNOS (inducible NO synthase) required for NO production is a bona fide marker of murine M1 Mϕs (Xie and Nathan, 1993). Other marker genes, whose expression is induced in M1, include *ido1*, *ptgs2*, *il12b/il23a*, *socs3*, *marco*, *cd86*, *irf3/irf5*, and *stat1/stat5*, among others (Lawrence and Natoli, 2011; Murray and Wynn, 2011b). Collectively, the M1 program is part of the "common host response" against intracellular bacteria that endows Mϕs with a non-permissible nature (Ehrt et al., 2001; Deretic et al., 2004; Martinez et al., 2009; Cairo et al., 2011; Murray and Wynn, 2011a). On the other hand, the M2 program is dictated by type-2 inflammatory signals (e.g., IL-4, IL-10), enabling Mϕs to participate in the suppression of inflammation, phagocytosis, tissue remodeling, and repair, among others (Sica et al., 2008; Martinez et al., 2009; Murray and Wynn, 2011a). However, this program also renders Mϕs poorly microbicidal against intracellular pathogens (Raju et al., 2008; Martinez et al., 2009). This is best illustrated by how the arginine metabolism is used in M2 Mϕs, which shuts down NO production in favor of tissue reparation (Shearer et al., 1997). Indeed, M2 polarization is accompanied by ARG1 (type-1 arginase) expression that inhibits NO production by outcompeting iNOS to convert arginine into ornithine and urea (Munder et al., 1998; El Kasmi et al., 2008). Along arg1, other M2 marker genes include *fizz1*, *chi311/chi312/chi313*, *mrc1*, *cd36*, *socs2*, *il-10*, *klf4*, *jmjd3/irf4*, *pparγ*, and *stat6*, among others (Lawrence and Natoli, 2011; Murray and Wynn, 2011b). Altogether, Mtb might influence the granuloma function by controlling Mϕ polarization, a premise that is presciently in line with the following findings, which for the purpose of conciseness, are mainly based on the use of the iNOS/ARG1 polarization axis.

The animal models to study TB granulomas are discussed in detail elsewhere (Flynn, 2006). Here, we highlight recent findings in mice and zebrafish documenting the TB granuloma dynamics, supported by studies and clinical observations done in TB patients. It is widely postulated the onset of human pulmonary TB begins when inhaled Mtb is captured by Mϕs and transported across the alveolar epithelium into the lung tissue. In zebrafish, the subsequent steps leading to a nascent granuloma have been captured in real-time imaging (Davis et al., 2002). While infected Mϕs undergo apoptosis, they promote the recruitment of phagocytes, which upon arrival, display high motility conducive for scavenging apoptotic cells. The phagocytosis of dead Mϕs leads to the formation of cell aggregates, fomenting bacterial growth. Subsequent rounds of this cycle promote the formation of a stable granuloma in 3 days post-infection (*p.i.*), a process that is dependent on the region of difference-1 (RD1) virulence locus of *M. marinum* and independent of T-cells (Davis et al., 2002; Volkman et al., 2004, 2010; Davis and Ramakrishnan, 2009). It is unclear whether zebrafish Mϕs undergo polarization. Yet, since most transcription factors governing T-cell polarization are highly conserved in zebrafish (Mitra et al., 2010), along with physiological and pathological responses characteristic of type-1 and type-2 immunity (Aggad et al., 2010; Balla et al., 2010; Holt et al., 2011; Wittamer et al., 2011; Renshaw and Trede, 2012), it seems as a matter of time before Mϕ polarization is identified and characterized in this teleost. By contrast, the early stage of Mtb infection in mice is marked by M1 Mϕ polarization, reminiscent of clinical observations in TB patients (Benoit et al., 2008). In fact, transcriptomic analysis of infected murine Mϕs revealed the gene modulation provoked by Mtb overlaps with that of IFN-γ to establish the M1 program (Ehrt et al., 2001). Type-1 inflammatory signals secreted by infected Mϕs induce cell recruitment and formation of primary granulomas. Unlike zebrafish, however, granuloma formation in mice takes up to 3 weeks when *Mycobacterium* reaches a plateau and coincides with adaptive immunity involvement. For instance, nascent liver granulomas were visualized by intravital microscopy between 2 and 3 weeks after *Mycobacterium bovis* Calmette–Guerin (BCG) challenge (Egen et al., 2011). In another study, Mtb infection did not change the murine Mϕ population ($iNOS^{low}ARG1^{low}$) in bronchoalveolar lavage (BAL) during the first week (Redente et al., 2010). At day 21 *p.i.*, however, M1 Mϕs ($iNOS^{high}ARG1^{low}$) dominated in BAL and granulomas, coinciding with a peak of IFN-γ in infected lungs (Redente et al., 2010). In humans, although NO production by monocyte-derived Mϕs remains controversial, both iNOS and NO are detected in granulomas and alleles for NOS2 are associated to TB susceptibility (Nicholson et al., 1996; Facchetti et al., 1999; Choi et al., 2002; Schon et al., 2004; Moller et al., 2009). After 35–60 days *p.i.*, while murine Mϕs at the granuloma core remained $iNOS^{high}ARG1^{low}$, there was a dramatic shift toward the M2 program ($iNOS^{low}ARG1^{high}$) in Mϕs surrounding the core, accompanied by elevated type-2 inflammatory signals (Redente et al., 2010). This is in line with ARG1 detection in human TB granulomas (Pessanha et al., 2012).

The shift toward M2 Mϕs during Mtb infection could have deleterious consequences for the granuloma as a host-protective structure (**Figure 1**). First, ARG1 expression in uninfected Mϕs surrounding the granuloma core suggests the development of an immunosuppressive niche. Indeed, Mtb promotes its survival by inducing ARG1 expression through MyD88-dependent signaling pathways (El Kasmi et al., 2008; Qualls et al., 2010). At the transcriptome level, murine M2 Mϕs displayed a diminished inflammatory response to Mtb as reflected by a reduced NO production and increased of iron availability, alluding ARG1 might also be implicated in nutrient deprivation mechanisms limiting microbial growth (Forbes and Gros, 2001; Kahnert et al., 2006; Cairo et al., 2011). Furthermore, M1 Mϕs possess a "fail-safe" system sustaining optimum NO production based on citrulline recycling via argininosuccinate synthase (ASS1), which is absent in M2 Mϕs (Qualls et al., 2012). Given the restrictive granuloma environment where arginine may be limited, the presence of this fail-safe system may become further accentuated. Second, M2 Mϕs may represent a transitional state into the formation of "foamy" Mϕs that are rich in cholesterol, a carbon source for microbial intracellular survival (Pandey and Sassetti, 2008; Peyron et al., 2008; Russell et al., 2009; Griffin et al., 2011). Recently, Mtb lipids were shown to trigger PPARγ, the master regulator of M2 polarization, to increase expression of CD36 and induce foam cell formation

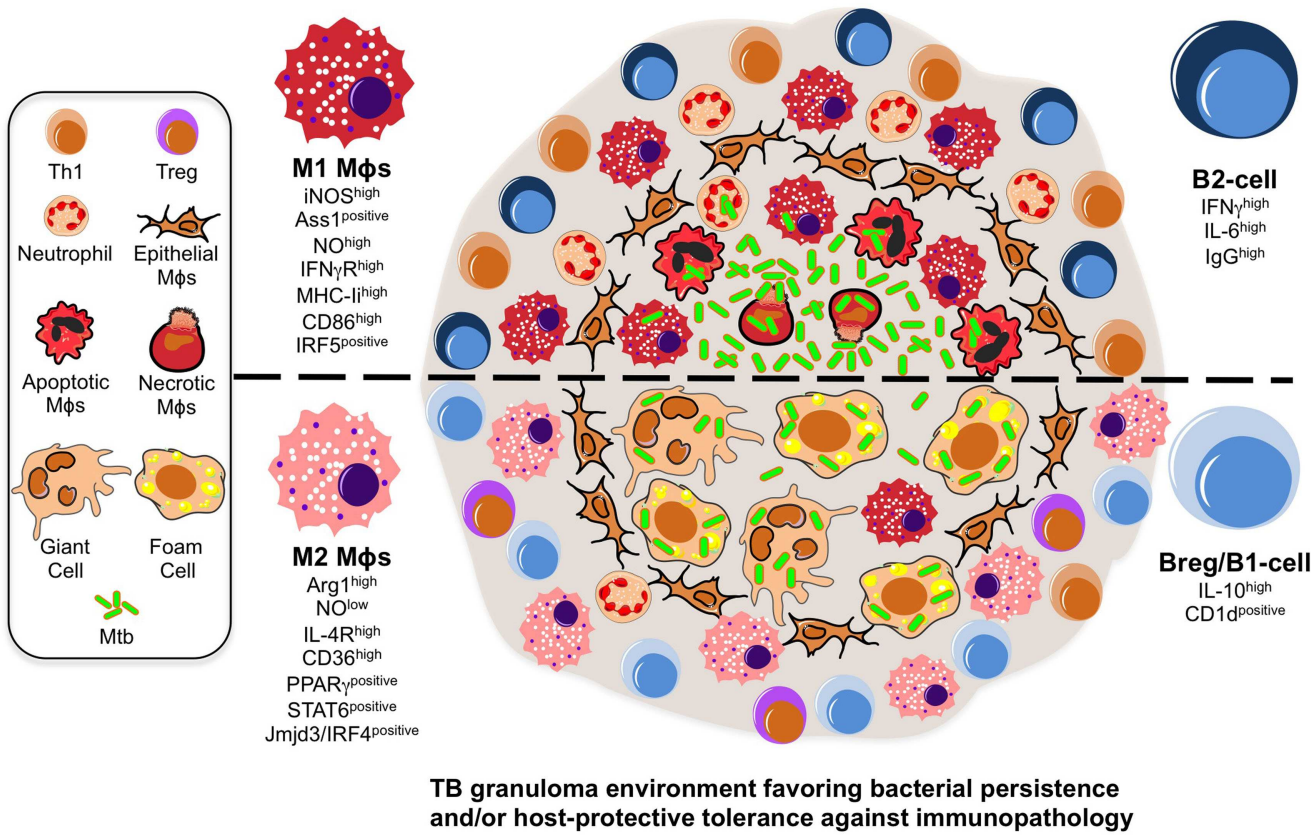

FIGURE 1 | A model illustrating the putative roles of Mϕ polarization and B-cell involvement during the formation and function of TB granulomas. TB granuloma Mϕs undergo various specialized transformations: they can look like epithelial characterized by tightly interdigitated cell membranes that link adjacent cells; they can fuse into multinucleated giant cells; or they can differentiate into foamy cells with a high content of intracellular lipids. While none of these specialized transformations in the granuloma Mϕ population are well understood, we propose they might be reflection of the Mϕ polarization status that may render the granuloma structure with a microbicidal capacity (top) or as a tool of pathogenesis (bottom). In the former scenario, the local Mϕ population in lung undergoes a M1 polarization early on during Mtb infection and granuloma formation, distinguished by a cell-surface receptor repertoire responsive to pro-inflammatory signaling (e.g., IFN-γR^{high}) and conducive for antigen-presentation (e.g., MHC-II^{high}, CD86^{high}), while acquiring a microbicidal capacity reflected in the NO production (e.g., *iNOS*^{high}, ASS1^{positive}), among others. These Mϕs have been noted to be most frequently located in the necrotic center of a mature tuberculous granuloma where apoptotic and necrotic Mϕs are abundant along with extracellular bacteria. Accompanying the M1 Mϕ polarization is the recruitment of neutrophils and Th1 cells, whose migration and activation status might be influenced by a B-cell involvement likely characterized by a pro-inflammatory phenotype (e.g., IFN-γ^{high} IL-6^{high} IgG^{high}). In the latter scenario, we propose a change in the TB granuloma environment during the late stages of Mtb infection, distinguished by the M2 Mϕ polarization driven by the high expression of transcription factors (e.g., PPARγ^{high}, STAT6^{positive}) antagonistic for type-1 inflammation, and characterized by a cell-surface receptor repertoire promoting tissue repair activities (e.g., IL-4R^{high}) and the formation of foamy cells (e.g., CD36^{high}), while suppressing the microbicidal functions like NO production (e.g., ARG1^{high}), among others. We envision M2 Mϕ polarization might give rise to the formation of foam and multinucleated giant cells, whose presence is noted to be most frequently at the rim and center of mature TB granulomas, and which may favor the intracellular resilience of Mtb. Furthermore, classical M2 Mϕs have been noted to be most frequently located surrounding the granuloma center and overwhelmingly in the local lung environment. Along with the M2 Mϕ polarization is the inhibition of neutrophil recruitment while enhancing that of T_{regs}, activities that might be influenced by a B-cell involvement likely characterized by a anti-inflammatory phenotype (e.g., IL-10^{high}, CD1d^{positive}).

(Mahajan et al., 2012). Here, we postulate that factors governing M2 polarization establish additional anti-inflammatory signaling loops, like that of CD36, to increase microbial fitness within granulomas (Kuda et al., 2011). Third, the shift toward M2 Mϕs may allow Mtb to control the antigen-presentation process to undermine adaptive immunity within granulomas (Benoit et al., 2008). Indeed, TB granulomas display a limited antigen-presentation to evoke significant T-cell responses (Egen et al., 2011). While Mϕ polarization was not addressed in this study, M2 Mϕs do inhibit the proliferation of CD4 T-cells while fomenting the activity of regulatory T-cells (Schebesch et al., 1997; Curiel et al., 2004; Biswas and Mantovani, 2010). Altogether, the shift toward M2 Mϕs might also occur in human granulomas and contribute to Mtb pathogenesis given that TB susceptibility is often accompanied by elevated

type-2 inflammatory and immunosuppressant signals (Kahnert et al., 2006; Raju et al., 2008; Almeida et al., 2009; Schreiber et al., 2009).

In the near future, we envision the role of Mϕ polarization in the granuloma context will be tested directly in different ways. First, we expect further advances in real-time imaging in both zebrafish and mouse models. Highly conserved Mϕ polarization markers are ideal candidates for the development of novel animal reporter lines expressing different fluorochromes to target the different Mϕ subsets. Second, specific gene inactivation of Mϕ polarization markers with the use of morpholinos (in zebrafish), siRNA-based technology, or gene-knockout strategy (including conditional strategies), may be used at different stages of granuloma formation in animal models. The strategies above could be used in combination with global array-based transcriptomics and proteomics approaches in order to assess the granuloma and local lung environment in the presence or absence of Mϕ subsets. Collectively, we expect there would be more future efforts to bridge results obtained in animals into the human context as discussed in the conclusion section.

A ROLE FOR B-CELLS IN GRANULOMATOUS DISEASES

Alterations in the lung environment by Mtb and/or subsequent immune responses likely affect the infection outcome. None of these is more apparent than the type-1 inflammatory storm that is unleashed in murine lungs at 3 week *p.i.*, when a peak of IFN-γ/TNF coincides with $CD4^+$ T-cell involvement, an event that impacts the organization of nascent granuloma structures. Yet, mice in which $CD4^+$ T-cells are unable to produce IFN-γ/TNF are still resistant to TB, suggesting a complex scenario for protection (Torrado and Cooper, 2011). In this perspective article, we propose that, beside T-cells, B-cells modulate the TB granuloma formation and function through interaction with their cellular components.

Despite extensive evidence for anti-Mtb antibody production in TB patients (Kunnath-Velayudhan et al., 2010, 2012), and a higher susceptibility of pIgR (IgA receptor)-deficient mice (Tjarnlund et al., 2006), initial studies examining the role of antibodies in TB indicated a modest impact in protective immunity, with benefits limited to passive administration of anti-Mtb antibodies (Glatman-Freedman and Casadevall, 1998; Roy et al., 2005; Abebe and Bjune, 2009). This contributed to the notion B-cells played a minor role in TB immunity, if any. Yet, recent studies now provide compelling reasons to revisit the role of B-cells in TB (Cooper, 2009; Maglione and Chan, 2009; Flynn et al., 2011; Philips and Ernst, 2012). First, B-cells infiltrate the lungs of Mtb-infected mice and humans (Tsai et al., 2006), where they organize in ectopic B-cell follicles at the periphery of granulomas (Gonzalez-Juarrero et al., 2001; Ulrichs et al., 2004; Kahnert et al., 2007; Maglione et al., 2007). These foci are the predominant sites of cellular proliferation in the infected lungs attesting to the importance of B-cells in shaping the local environment during infection (Ulrichs et al., 2004). Moreover, B-cells also infiltrate the granuloma structure, as shown in non-human primates where activated B-cell clusters are found in close contact with T-cells (Phuah et al., 2012), and in the lungs from cattle with natural tuberculosis (Beytut, 2011). Mtb-specific B-cells also exist at local sites of infection in pleural fluids, a strategic place to influence the immunity against Mtb (Feng et al., 2011). Beyond TB, B-cells are well-known cellular components in

Table 1 | Characteristics of B-cells identified in non-TB granulomatous diseases.

Disease or model	Type of B-cells	Reported role in disease	Specie	Reference
Wegener's granuloma	Undefined	Detrimental	Humans	Voswinkel et al. (2008), Holle et al. (2012)
Sarcoidosis	Undefined	Unknown	Humans	Fukuda et al. (1997)
Churg–Strauss syndrome	Undefined	Detrimental	Humans	Donvik and Omdal (2011)
Crohn's disease	B1[a]	Unknown	Humans	Geboes et al. (1986)
Schistosomiasis	Undefined	Favor protective Th2 immunity; inhibit T-cell-mediated immunopathology; granuloma formation	Mouse	Hernandez et al. (1997, Ferru), Jankovic et al. (1998), Jacobs et al. (1999), Ji et al. (2008)
Leishmaniasis	Include B2[b] as well as $CD5^+CD1d^+$IL-10 producing regulatory Breg[c] cells	Limits immunopathology; favor protective Th2 immunity; favor granuloma formation	Mouse	Smelt et al. (2000), Ronet et al. (2010), Moore et al. (2012)
Coccidioidomycosis	IL-10 producing B_{regs}	Unknown	Humans	Li et al. (2005)
Paracoccidiois	B1, IL-10 producing B_{regs}	Detrimental	Mouse	Popi et al. (2008)
Cat-scratch disease	IL-10 producing B_{regs}	Unknown	Humans	Vermi et al. (2006)
Pristane induced oil granuloma response	Undefined	Granuloma formation	Mouse	Chen et al. (2010)

[a] *B1 cells: developmentally defined; innate-like B-cells in the mouse; $CD5^+$ or $CD5^-$ subpopulation poorly defined in humans.*

[b] *B2 cells: developmentally defined; include "conventional" follicular B-cells as well as "innate-like" marginal zone B-cells.*

[c] *B_{regs}: functionally defined; present among various B-cell populations including $CD5^+CD1d^+$ B-cells; can produce IL-10.*

several other granulomatous diseases (**Table 1**). Not only B-cells are present in granuloma but also they could be important for their maturation. This is suggested in pristane induced oil granuloma formation (Chen et al., 2010) and during *Schistosoma japonicum* infection (Ji et al., 2008) where the absence of B-cells results in a marked delay in granuloma formation. In the context of the TB, although granulomas form in the absence of B-cells, their numbers and size remain lower and they hardly become inflammatory (Bosio et al., 2000; Maglione et al., 2007). This could be the result of the well-known ability of B-cells to contribute to the organization of secondary and tertiary lymphoid organs (Moseman et al., 2012).

Second, although this is a rare event, occurrence of mycobacterial infections was reported upon rituximab-mediated depletion of B-cells, suggesting a protective role for these lymphocytes (Winthrop et al., 2008; Gea-Banacloche, 2010). However, other granulomatous diseases were successfully treated with rituximab (Donvik and Omdal, 2011; Holle et al., 2012), cautioning B-cells may be detrimental depending on the disease context. Finally, beyond antibody production, B-cells display diverse roles in the immunity against multiple pathogens that could operate during TB. In this regard, *Salmonella* infection, though not occasioning granuloma formation, represents a paradigm for antibody-independent roles of B-cells against an intracellular bacterium with the evidence that B-cells producing IL-10 (B_{regs}) impairs the control of natural and vaccine-induced immunity to *Salmonella* (Neves et al., 2010). Since this role cannot simply be recapitulated in animal models lacking B-cells (Mastroeni et al., 2000; Mittrucker et al., 2000), this exemplifies how deletion of the B-cell compartment eclipses specific functions of these cells.

B-cells express adaptive and innate receptors to recognize pathogens (Blumenthal et al., 2009; Rawlings et al., 2012). Beyond antibody production, B-cells secrete various signals including cytokines, and serve as antigen-presenting cells (Rawlings et al., 2012). These immune-modulatory functions are performed by different B-cell subsets depending on their differentiation program (e.g., B1, B2), activation status (e.g., naïve, effector, memory), tissue distribution, the timing of the immune response, or disease context. From this perspective, the identity of B-cells infiltrating the lungs of TB patients or animals remains relatively unknown. In most cases, these cells (likely B2-cells) have undergone class switch recombination and produce antibodies (Phuah et al., 2012). However, $CD5^+CD1d^+$ B1-cells are also observed predominantly in TB patients (Zhang et al., 2012) and in mouse models of TB and other granulomatous diseases (Li et al., 2005; Popi et al., 2008; Ronet et al., 2010). Regardless of their identity or individual contribution, we estimate the B-cell compartment influences the TB granuloma formation and function through interaction with Mϕs, T-cells, and neutrophils (**Figure 1**).

As B-cells interact with Mϕs in TB granulomas (Tsai et al., 2006; Chakravarty et al., 2008), they might affect Mϕ polarization within these structures. A case in point, B1-cells differentiate M2 Mϕs via IL-10 *in vitro* and in a tumor model (Wong et al., 2010). However, mice deficient for B1-cells (xid model) displayed rather a susceptibility to mycobacterial infection, accompanied by increased levels of IL-10 (Junqueira-Kipnis et al., 2005; Russo and Mariano, 2010). Certainly, there are other B-cell subsets that could compensate as the *in vivo* source of IL-10, like B_{regs} (O'Garra et al., 1990; Lampropoulou et al., 2008). Likewise, there exist alternative *in vivo* immunosuppressive mechanisms driven by B-cells other than the B1-cell subset, as demonstrated for IgG production favoring FcR-mediated M2 Mϕ polarization in a carcinoma model (Andreu et al., 2010). In line with this observation, FCγRIIB-deficient Mϕs displayed a M1 Mϕ phenotype upon Mtb infection, express less IL-10 and better control the infection (Maglione et al., 2008). Since the phenotype manifests after 3 weeks of infection, IgG-producing B2-cells produced during the course of the adaptive immune response might be involved. B1 cells might rather contribute to M2 polarization through FcγR-independent IL-10-dependant mechanisms. Whether these events occur within the granuloma is currently unknown. Collectively, these studies infer a B-cell contribution to an immunosuppressive niche within TB granulomas by tilting Mϕs toward the M2 program.

If Mϕs are the main components in nascent TB granulomas, then $CD4^+$ T-cells are perhaps the most critical component of stable TB granulomas as shown by the re-awakening of latent TB in HIV-1 co-infected patients. In recent years, multiple studies suggest an immune-modulatory role for B-cells in T-cell activity at the granuloma level. On one hand, B-cells can co-localize with T-cells in TB granulomas (Ulrichs et al., 2004; Beytut, 2011), and directly interact with them in the granulomas caused by *Leishmania* (Moore et al., 2012). On the other hand, B-cells influence T-cell effector functions either through cytokine production or antigen-presentation (Lund and Randall, 2010). In TB context, IL-10 derived from B1-cells controls the homeostasis of T-helper-17 (Th17), essential for anti-microbial immunity at epithelial/mucosal barriers (Zhang et al., 2012). Reciprocally, Th-17-associated cytokines promote the formation of B-cell foci in Mtb-infected mice, and correlate with B-cell infiltration in TB patients (Khader et al., 2011; Zhang et al., 2011). In the mouse model, IL-17A (Okamoto Yoshida et al., 2010) or IL-23-deficient (Khader et al., 2011; Zhang et al., 2011) animals have marked defects in the formation of granulomas and/or B-cell follicles. In addition IL-23-deficient mice also have poor levels of IL-17 and IL-22. These deficiencies resulted in a marked alteration of CXCL13 production, the chemokine responsible for B-cell recruitment and follicle formation (Khader et al., 2011; Zhang et al., 2011). It is not known if IL-10 production by B-cells is at the initiation or a secondary consequence of the alterations in IL-17 levels. These observations might provide an explanation for the links reported in TB patients between Th17 and formation of B-cell foci and IL-10 (Zhang et al., 2011, 2012).

Evidence obtained in non-TB diseases argue B-cells favor Th1 polarization (involved in TB protective immunity) through IL-6 and IFN-γ production during *Salmonella* infection, or promote Th2 differentiation (thought to be detrimental during TB) through either IL-2 (Wojciechowski et al., 2009) or IL-10 (Ferru et al., 1998; Popi et al., 2008; Ronet et al., 2010) in the control of different parasites. Conversely, B-cells also suppress T-cell activity as best illustrated in mice with a targeted deletion of MyD88 in B-cells during *Salmonella* infection (Neves et al., 2010). Finally, evidencing the role of B-cells as antigen-presenting cells, mice with a targeted deletion of MHC-II in B-cells displayed a reduction of IL-2 and IFN-γ by $CD4^+$ memory T-cells during

Salmonella challenge (Barr et al., 2010), and low pulmonary Th1 cell counts during *Pneumocystis* infection (Lund and Randall, 2010).

Another cell influencing TB granuloma formation is the neutrophil, whose migration can be controlled by B-cells. During *Salmonella* infection, mice with a targeted deletion of MyD88 in B-cells exhibited an accumulation of neutrophils in the spleen, an effect that likely depends on B_{regs}-mediated IL-10 production (Neves et al., 2010). In the context of mycobacterial infections, aberrant neutrophil migration is known to have deleterious effects in host tissue integrity (Eruslanov et al., 2005; Berry et al., 2010). In mice deficient for the B-cell compartment (Maglione et al., 2007), Mtb infection leads to an uncontrolled accumulation of pulmonary neutrophils, an observation also supported by the excessive neutrophil migration in the peritoneum after BCG-vaccination (Kondratieva et al., 2010). These examples highlight the importance of tolerance mechanisms in TB.

Based on the above observation, it is tempting to propose that B-cell could act at different levels during TB such as during granuloma progression and by influencing the effector function of third-party cells like Mϕs. To directly examine this, studying B-cell contribution through comparison of B-cell-competent vs. B-cell-deficient animals should now be further complemented by studies examining the direct response of B-cells to Mtb infection, and through analyses in animal models lacking specific pathways in B-cells and biological consequences.

CONCLUSION

Among trends emerging in TB etiology, the notion that the local lung environment shifts from a host-protective nature toward one favorable to microbial resilience is discussed here at the granuloma level and in the context of Mϕ polarization and B-cell function (see also an illustration in **Figure 1**). Exploring these issues will likely bring us closer to uncover the enigma concealed by TB granulomas. One can envisage that studies investigating the role of genes involved in host tolerance (Medzhitov et al., 2012) might be a good way to explore these aspects of the disease. Although in humans this could be limited to immunogenetic studies, more mechanistic studies could be conducted in animal models where selective inactivation of those genes could provide new insights on the consequences on the pathology. These studies could go along with more sophisticated approaches based on single cell analysis such as those involving laser microdissection or more global phenotypic signatures obtained from mass cytometry, in order to further identify cell subsets involved at different stages of granuloma formation and TB.

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Recognition of the mycobacterial cord factor by Mincle: relevance for granuloma formation and resistance to tuberculosis

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The world's most successful intracellular bacterial pathogen, *Mycobacterium tuberculosis* (MTB), survives inside macrophages by blocking phagosome maturation and establishes chronic infection characterized by the formation of granulomas. Trehalose-6,6-dimycolate (TDM), the mycobacterial cord factor, is the most abundant cell wall lipid of virulent mycobacteria, is sufficient to cause granuloma formation, and has long been known to be a major virulence factor of MTB. Recently, TDM has been shown to activate the Syk-Card9 signaling pathway in macrophages through binding to the C-type lectin receptor Mincle. The Mincle-Card9 pathway is required for activation of macrophages by TDM *in vitro* and for granuloma formation *in vivo* following injection of TDM. Whether this pathway is also exploited by MTB to reprogram the macrophage into a comfortable niche has not been explored yet. Several recent studies have investigated the phenotype of Mincle-deficient mice in mycobacterial infection, yielding divergent results in terms of a role for Mincle in host resistance. Here, we review these studies, discuss possible reasons for discrepant results and highlight open questions in the role of Mincle and other C-type lectin receptors in the infection biology of MTB.

Keywords: mycobacteria, cord factor, TDM, Mincle, C-type lectin receptor, tuberculosis

TDM's HISTORY AS PAMP AND GLYCOLIPID EFFECTOR MOLECULE OF MYCOBACTERIA

Trehalose-6,6-dimycolate (TDM) has been isolated as a major glycolipid from the cell wall of pathogenic mycobacteria in the 1950s by Bloch and colleagues (see Hunter et al., 2006 for review). The biological properties of TDM have been explored *in vivo* using mice and rabbit models and led to the realization that TDM is sufficient to induce formation of granulomas when injected in oil doplets (Yarkoni and Rapp, 1977; Ishikawa et al., 2009). These data indicated that TDM *per se* triggers an important reaction to mycobacterial infection, which has traditionally been viewed as a correlate of protective immune responses, required to wall off infection. TDM also possesses adjuvant properties and is contained in the experimental adjuvant Ribi (Gavin et al., 2006). While these properties of TDM indicated that it acted as a pathogen-associated molecular pattern (PAMP), indicating microbial danger and alerting innate immune cells, the cord factor has also been recognized since so long as a virulence factor of mycobacteria (**Figure 1**). First, extraction of glycolipids with petroleum ether from the cell wall of MTB rendered the mycobacteria avirulent, due to efficient killing of the TDM-less mycobacteria inside macrophages (Indrigo et al., 2002, 2003). Second, when replacing this rough and crippling procedure with genetic ablation of TDM biosynthesis, the attenuated phenotype was confirmed in ΔfbpA mutants lacking the mycolic acid transferase antigen 85A (Katti et al., 2008). In addition, these mutants triggered enhanced production of inflammatory cytokines and increased antigen presentation from infected macrophages (Kan-Sutton et al., 2009). Third, TDM coated onto beads is sufficient to delay phagosome maturation in macrophages (Axelrod et al., 2008), whereas the mutant MTB lacking TDM failed to block phagosome maturation (Katti et al., 2008). Together, the cord factor qualifies as a glycolipid effector molecule of pathogenic mycobacteria that reprograms macrophages for mycobacterial immune evasion and creation of a niche in the phagosome.

TDM AS PAMP

A ROLE FOR TLR?

Despite the decade-long knowledge of the inflammatory capacities of the cord factor, the mechanisms of immune cell activation by TDM were elusive until recently. As an abundant mycobacterial cell wall glycolipid with a chemical structure not found in vertebrate organisms, TDM *a priori* qualifies as a prototypical PAMP that may signal the presence of mycobacterial danger to innate immune cells. The phenocopying of inflammatory and granulomatous responses-induced by whole mycobacteria by TDM in animal models, and its long known property as an adjuvant eliciting cellular immune responses to co-administered protein antigen, support the notion of TDM being a PAMP. After the discovery of the toll-like receptor (TLR) family and several mycobacterial ligands that are recognized by TLR family members (ManLAM, 19 kDa lipopeptide), the search for a pattern recognition receptor sensing TDM naturally first focused on a potential role of TLR. In Geisel et al. (2005) reported that TDM-coated beads stimulated TNF and IL-6 production by macrophages

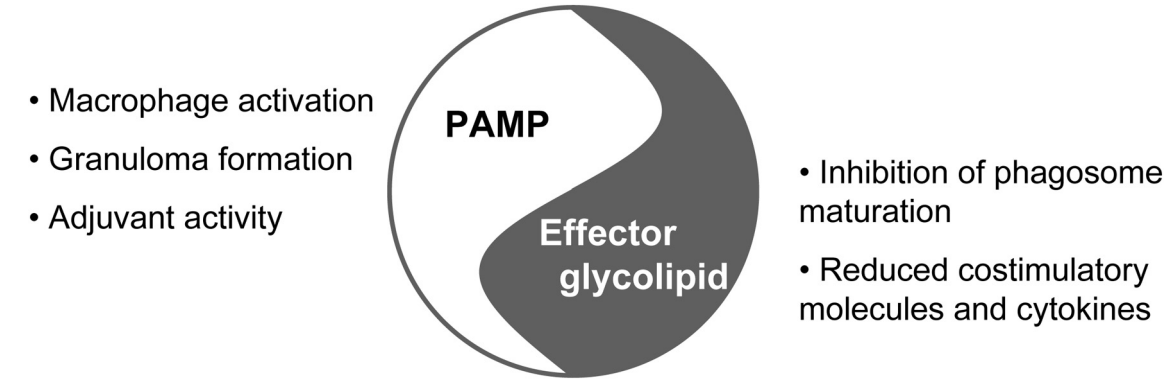

FIGURE 1 | TDM between PAMP and effector molecule.

in vitro, and that TDM in gel matrices injected s.c. into mice triggered massive leukocyte infiltration (Geisel et al., 2005). The role of TLR2 and TLR4, and of the adapter protein Myd88, was investigated exclusively for the *in vivo* leukocyte recruitment but not for the macrophage cytokine response *in vitro*. While TLR2$^{-/-}$ and TLR4$^{-/-}$ mice had no defect in leukocyte recruitment to TDM gel matrices, Myd88-deficient mice showed a severe reduction, pointing to a possible role for other TLRs or of other Myd88-utilizing receptors, e.g., for the cytokines IL-1 or IL-18. This initial report from the Russell lab was followed by a study reporting that murine macrophages deficient in TLR2 and in TLR4 had a strongly reduced response to TDM (Bowdish et al., 2009). In addition, the scavenger receptor MARCO was suggested to bind TDM in this study. Since scavenger receptors lack a cytoplasmic domain and are usually endocytic clearance receptors rather than activating PRR, a model was proposed where MARCO binding of TDM serves as a prerequisite for delivery to and activation of TLRs (Bowdish et al., 2009). In this context, a previous study is also of interest that investigated macrophages lacking Scavenger receptor A (SR-A) *in vitro* and observed an increased inflammatory response; importantly, these authors reported also that SR-A bound to the cord factor (Ozeki et al., 2006).

TDM ACTIVATES MINCLE-Syk-Card9 SIGNALING

Our own studies first aimed at determining a role of the TLR pathway for macrophage and DC activation-induced by TDM and its synthetic analog TDB. We observed that bone marrow derived macrophages from mice lacking Myd88 responded comparably to wild type cells (Werninghaus et al., 2009). In addition, the adjuvant activity of the cord factor analog TDB was preserved in mice deficient in TLR2, TLR3, TLR4, and TLR7 (Agger et al., 2008). This apparent lack of TLR-dependence led us to consider other PRR pathways. At this time, the C-type lectin receptor Dectin-1 (Brown, 2006) had just been described to activate macrophages via Syk-Card9 signaling (Gross et al., 2006). We employed macrophages from the respective KO mice to genetically define that macrophage activation by TDB and TDM requires Syk-Card9-Bcl10-Malt1 signaling, but is independent of Dectin-1 (Werninghaus et al., 2009). In contrast to Dectin-1 with its intracellular non-classical ITAM motif, other CLR activate Syk *via* association with an adapter protein carrying the classical ITAM motif (Robinson et al., 2006). The requirement for the adapter protein FcRg but not Dap12 pointed to a certain set of C-type lectin receptors, including the family member Mincle (gene symbol: Clec4e) that was expressed inducibly in macrophages stimulated with the glycolipids. Mincle had been described in 2008 by Yamasaki et al. as sensor of necrosis that binds SAP130, a splicing factor released from dying cells (Yamasaki et al., 2008). Using independent lines of Mincle KO mice, Yamasaki et al. and Wells et al. demonstrated a function of Mincle in the recognition of fungal carbohydrates in the cell wall of Candida and Malassezia yeast (Wells et al., 2008; Yamasaki et al., 2009). A role for Mincle in recognition of mycobacteria was suggested by Yamasaki's group through experiments using a Mincle-reporter cell line. In a series of elegant experiments, they then identified the TDM from cell wall lipid fractionations as the active compound, showed direct binding of Mincle to TDM, and demonstrated that Mincle$^{-/-}$ mice fail to produce the granulomatous response to TDM observed in the lungs of wild-type mice (Ishikawa et al., 2009). In parallel, experiments with Mincle-deficient mice in our lab showed that TDB and TDM do not activate macrophages from these mice anymore and that the Th1/Th17 adjuvant effect of TDB requires Mincle *in vivo* (Schoenen et al., 2010). Together, these studies provided solid evidence that Mincle is an essential receptor for the mycobacterial cord factor (Matsunaga and Moody, 2009) (**Figure 2**). Until now, however, the requirement for Mincle in TDM recognition has only been shown in the mouse system, and the possibility that the response to the cord factor in human macrophages is mediated by the same receptor and pathway remains to be proven experimentally (Lang et al., 2011).

POTENTIAL FOR SYNERGY AND CROSS-REGULATION OF TLR AND MINCLE SIGNALING

The reasons for the discordant results concerning the involvement of TLR-Myd88 signaling for TDM recognition between the Bowdish et al. (2009) and our studies (Werninghaus et al., 2009; Schoenen et al., 2010) are at present unclear. Differences in the type of macrophage used (bone marrow derived vs. resident peritoneal macrophages), culture conditions, and the presentation of

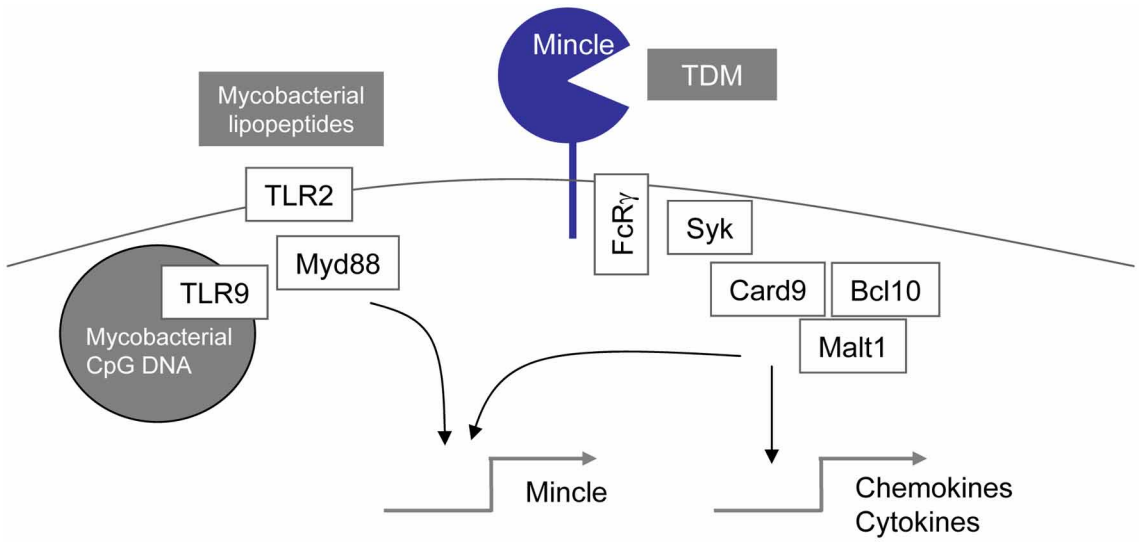

FIGURE 2 | TDM recognition and responses through regulated Mincle expression: possible synergy with TLR signals.

TDM (coated onto the cell culture dish vs. presented on beads) may be responsible. In general, TLR and CLR pathways can synergize to activate gene expression in macrophages. This has been clearly shown in the case of zymosan-triggered activation of macrophages through Dectin-1 and TLR2 (Dennehy et al., 2008, 2009). In the case of Mincle-mediated recognition of the cord factor, the inducible expression of the Mincle receptor following stimulation with TLR ligands (Matsumoto et al., 1999) suggests a mechanism of priming of macrophages for increased sensitivity to the CLR ligand TDM (**Figure 2**). The role of the MARCO-TLR2-Myd88 pathway described by Bowdish et al. for TDM recognition (Bowdish et al., 2009) may therefore consist in licensing of macrophages for TDM responsiveness through induction of Mincle expression. A synergistic effect of Myd88 and Mincle for TDM responsiveness has very recently been shown for neutrophils by Lee et al. (2012): Myd88$^{-/-}$ neutrophils responded equally well with TNF release and adhesion to TDM as WT; in contrast, upon combined treatment with the TLR2 ligand Pam3CSK and TDM, synergistic induction was observed in WT but not Myd88$^{-/-}$ neutrophils, creating a significantly reduced response in Myd88$^{-/-}$ vs. wild-type neutrophils that correlated with a lack of up-regulation of Mincle in the Myd88$^{-/-}$ cells (Lee et al., 2012).

IS MINCLE REQUIRED FOR ANTI-MYCOBACTERIAL IMMUNITY? CONTRADICTORY ANSWERS FROM MOUSE INFECTION MODELS

While the identification of Mincle as receptor for the cord factor solved a longstanding issue in TB research and provided a molecular basis for the adjuvant effect of TDM and TDB, the medically most important question remains whether Mincle and its associated pathway are required for anti-mycobacterial immunity, or may to the contrary be used by the pathogen to subvert immune responses.

In vitro STUDIES WITH PHAGOCYTES

The first steps taken to address the importance of the Mincle-Syk-Card9 pathway in the dealing of innate immune cells with infecting mycobacteria were *in vitro* infection experiments of macrophages with M. bovis *BCG* or MTB. These experiments showed a differential requirement for Mincle-signaling in the expression of several genes-induced by mycobacterial infection. Mincle-deficient macrophages expressed 10-fold less G-CSF and IL-6 in response to BCG (Schoenen et al., 2010). More recently, Behler et al. confirmed the essential role of Mincle for inflammatory cytokine and chemokine production in response to BCG in alveolar macrophages (Behler et al., 2012). Ishikawa et al. observed a slight reduction in the levels of several chemokines in Mincle$^{-/-}$ macrophages infected with MTB; this dependence on Mincle was strongly enlarged when macrophages from a Myd88$^{-/-}$ background were used (Ishikawa et al., 2009). Thus, a picture emerges that in the absence of Mincle macrophages can still sense mycobacteria through recognition of PAMPs other than TDM, but the inflammatory response is compromised at least for a subset of response genes. To date no published data is available that has tested whether phagocytosis of mycobacteria is reduced or delayed in Mincle$^{-/-}$ macrophages.

In vivo MYCOBACTERIAL INFECTION MODELS

To date, three publications have reported on experimental mycobacterial infection in Mincle$^{-/-}$ mice (Behler et al., 2012; Heitmann et al., 2012; Lee et al., 2012) (see **Table 1**). Heitmann et al. used aerosol infection with MTB H37Rv and comprehensively analysed bacterial burden in lung and spleen, as well as the inflammatory and T cell responses in the lungs and draining lymph nodes at three different time points after infection. In addition, histopathological analysis of the lungs for the extent of infiltration, granulomatous response and immunocytochemistry for iNOS expression was performed. None of these readouts

Table 1 | C-type lectin receptors in mycobacterial infection.

	Dectin-1	Mincle	Signr3
Mycobacterial ligand	Unknown	TDM	ManLAM
Expression of CLR	Macrophages and DC	Inducible in macrophages and DC; High constitutive expression in granulocytes	Up-regulated in alveolar macrophages during infection
Phenotype of knockout mouse in mycobacterial infection	Slightly reduced bacterial load in the lung; Unchanged adaptive immune response	Increased bacterial dissemination after BCG infection (Behler et al., 2012); Increased pulmonary load after MTB Erdman (Lee et al., 2012); Unchanged resistance after MTB H37Rv (Heitmann et al., 2012)	Increased mycobacterial load early in infection; Intact adaptive immune responses
References	Marakalala et al. (2011)	Behler et al. (2012); Heitmann et al. (2012); Lee et al. (2012)	Tanne et al. (2009)

showed a significant difference between Mincle$^{-/-}$ and WT control mice, leading to the conclusion that Mincle is not essential for the host response and protection against TB infection in mice. Low-dose aerosol infection with MTB is arguably the best mouse model for human TB; however, there are examples of knockout mice where the deletion of a gene did not cause a phenotype in the low dose model, but mice showed higher bacterial burden when challenged with a higher dose or by a different route (Reiling et al., 2002). To test for this possibility, Heitmann et al. also challenged Mincle$^{-/-}$ mice with a high dose of MTB H37Rv, again with no discernible difference in the bacterial load.

The second study reporting on the phenotype of Mincle$^{-/-}$ mice in MTB is the paper by Lee et al., who focused their interest on the expression and function of Mincle in neutrophilic granulocytes during *in vivo* and *in vitro* encounter with TDM (Lee et al., 2012). Corroborating and extending the results obtained by Yamasaki's group (Ishikawa et al., 2009), they showed that Mincle$^{-/-}$ mice fail to mount a granulomatous response to intravenously injected TDM and that Mincle expression on neutrophils is required for recruitment to TDM-coated beads injected subcutaneously. They further demonstrate that neutrophils attach to plate-bound TDM *in vitro* and respond in a Mincle-dependent manner with up-regulation of integrins and production of reactive oxygen species. These results are interesting because they draw attention to neutrophils which express higher levels of Mincle than macrophages and can immediately respond to TDM. Lee et al. also demonstrate that depletion of neutrophils with an anti-Ly6G antibody reduced the initial granulomatous response and the levels of IL-6 and MCP-1 in the lungs of TDM-injected mice. The analysis of MTB infection in the Mincle$^{-/-}$ mice in the study by Lee et al. consists of one low-dose aerosol infection experiment where bacterial loads in the lungs were determined at two time-points after infection, showing a significant but moderate increase (0.5 $\log_{10}$). The increased mycobacterial numbers in the Mincle$^{-/-}$ mice were paralleled by a stronger expression of inflammatory genes in the lungs. Given the expression and function of Mincle in granulocytes described in the same paper, it would be interesting to know whether neutrophil accumulation, integrin expression and cytokine production in the lungs is particularly affected in the mice infected in the Lee et al. study. The higher expression of inflammatory genes in Mincle$^{-/-}$ lungs is difficult to interpret because it could be entirely due to the increased mycobacterial load generating more PAMPs for activation of receptors other than Mincle. One can only speculate at which time after infection Mincle$^{-/-}$ mice start to have higher MTB burden in the lung; one possibility is that a lack of recruitment and proper activation of neutrophils early after infection gives MTB the ability to survive and grow better than in WT mice.

In contrast, the third study, reported by Behler et al., used the vaccine strain *M. bovis* BCG in intratracheal and intravenous infection models (Behler et al., 2012). These authors first characterized the expression of Mincle on alveolar macrophages following exposure to BCG *vivo*, and observed a strong up-regulation of receptor levels on the cell surface. This is consistent with previous observations *in vitro* at the mRNA and protein level (Yamasaki et al., 2008; Werninghaus et al., 2009; Schoenen et al., 2010), and importantly it first demonstrates that this process occurs during mycobacterial encounter *in vivo*, implying that Mincle induction by mycobacterial signals could serve to bolster recognition and defense against spread of mycobacteria or secondary challenge by subsequent exposure. In fact, this is exactly what the authors found in their further experiments: when mice were infected twice with BCG separated by 14 days, the inflammatory response to BCG in the airways and in alveolar macrophages strongly increased in a Mincle-dependent fashion. More importantly, the priming infection increased the Mincle-dependence of BCG-growth restriction, showing that the up-regulation of Mincle during the priming period indeed led to better innate control of intruding mycobacteria by alveolar macrophages. Another interesting effect of Mincle-deficiency in this study is the stronger increase in the bacterial burden in the draining lymph nodes and the spleen, compared to the lungs after intratracheal BCG infection (Behler et al., 2012). Dissemination of BCG after airway infection requires time until pulmonary mycobacteria have sufficiently expanded, allowing the quite slow induction of Mincle on alveolar macrophages (and also on other recruited cells, e.g., neutrophils) to generate the higher capacity of BCG recognition and

to mount a better response leading to containment of infection at the local site.

Taken together, these recent studies on the role of Mincle in the host response to mycobacterial infection yielded remarkably different results, raising the question for possible reasons that underlie the discrepant findings.

First, the three studies used different strains of *M. tuberculosis* (H37Rv in the Heitmann et al., Erdman in the Lee et al. study) or employed the vaccine strain *M. bovis* BCG (Behler et al.). Although all these strains have abundant TDM in the cell wall, they may differ in the length of mycolic acid chains, modifications and the mixture of other glycolipids present in the cell wall, which may account for the difference in the requirement for Mincle. Of interest, *in vitro* infection experiments of macrophages showed a stronger dependence of the NO production and of G-CSF secretion on Mincle when BCG was used compared to MTB H37Rv (Heitmann et al., 2012), which appears consistent with the larger role of Mincle in BCG vs. MTB H37Rv infection.

The relative amount of TDM present in the mycobacterial cell may also be affected by glycolipid exchange through the action of mycolyltransferases. Increased levels of glucose, as they are encountered by the mycobacteria in the cellular growth environment, can lead to a shift in glycolipid synthesis from TDM to glucose monomycolate (GMM) in an Ag85A-dependent manner (Matsunaga et al., 2008). GMM is a ligand for CD1b in humans (Moody et al., 1997), it has not been formally tested for binding to and activation of Mincle.

In principle, another variable can be introduced by changes in the culture conditions used to grow the mycobacteria before infection. For example, the commonly used inclusion of detergents like Tween in the mycobacterial broth to prevent clumping has been shown to drastically modify the presence of a polysaccharide capsular structure (Sani et al., 2010) and may also wash out TDM from the mycobacterial cell wall. Indeed, the inclusion of Tween in ELISA wash buffers can even remove coated glycolipids from microtiter plate plastic (Julian et al., 2001) and drastically reduce the detection of TDM-specific antibodies from the sera of TB patients (Traunmuller et al., 2005). It is therefore conceivable that inter-strain differences and subtle changes in the culture conditions used can significantly affect the composition of the mycobacterial cell wall and capsule, thereby affecting the interaction with Mincle and other pattern recognition receptors and changing the outcome of infection.

Finally, the hygiene status of mouse colonies can be expected to alter the basal levels of innate immune activation in general and of Mincle expression levels on phagocytes in particular through effects of TLR- and CLR-dependent PAMPs.

ROLE OF OTHER C-TYPE LECTIN RECEPTORS IN ANTI-MYCOBACTERIAL IMMUNITY

The adapter protein Card9 is expressed in myeloid cells and was initially identified as crucial for transducing the signal of the C-type lectin receptor Dectin-1 to NFkB activation (Gross et al., 2006). Card9 is down-stream of Syk and becomes phosphorylated by PKC delta (Strasser et al., 2012). Card9 is not only required for Dectin-1 signaling, but is also the central adapter for DAP12- and FcRγ-chain-coupled C-type lectin receptors like Mincle, Dectin-2 and Clec5a (Hara et al., 2007; Yamasaki et al., 2008; Werninghaus et al., 2009). Dorhoi et al. showed that the absence of Card9 renders mice exquisitely sensitive to infection with MTB (Dorhoi et al., 2010). Following aerosol infection, Card9$^{-/-}$ mice develop high mycobacterial burden in the lungs and die within 4–6 weeks. Card9$^{-/-}$ macrophages normally phagocytosed and killed MTB *in vitro*, but were impaired in the production of inflammatory cytokines and chemokines. *In vivo*, Card9$^{-/-}$ mice had a striking increase in neutrophil infiltration in the lung that was linked to tissue destruction and death of the mice by depletion experiments (Dorhoi et al., 2010). This essential role of Card9 in anti-mycobacterial defense, together with the relative lack of a phenotype in Mincle-deficient mice, poses the question which other C-type lectin receptors signaling through Card9 are triggered during MTB infection (see **Table 1**).

Dectin-1 has been implicated by several labs in the response to mycobacteria, but the nature of the ligand has remained unknown to date. Blocking Dectin-1 with antibodies or laminarin reduced cytokine responses to *M. bovis* BCG or MTB (Yadav and Schorey, 2006; Rothfuchs et al., 2007). In addition, a reduced uptake of *M. abscessus* and impaired ROS production was reported when Dectin-1 was blocked in mouse macrophages (Shin et al., 2008). To test the contribution of Dectin-1-mediated recognition of mycobacteria *in vivo*, Brown and colleagues challenged Dectin-1 KO mice with virulent MTB. The mycobacterial load in the lungs was unexpectedly slightly reduced in Dectin-1 KO mice, but there were no significant differences in lung pathology or adaptive immune responses (Marakalala et al., 2011). Thus, Dectin-1 appears to play a rather redundant role for protection against MTB.

The human C-type lectin DC-SIGN has received considerable attention in the mycobacterial immunity field when it was discovered that binding of mycobacterial Man-LAM to DC-SIGN on human macrophages down-regulates costimulatory molecules and inflammatory responses, presumably *via* induction of IL-10 production (Geijtenbeek et al., 2003). There are several murine homologues of human DC-SIGN in a cluster of seven genes and one pseudogene (Tanne et al., 2009). Based on the binding to glycans with high mannose content and fucose-containing oligosaccharides, mouse SIGNR3 is the best candidate as ortholog for human DC-SIGN. Tanne et al. created knockout mice for Signr1, Signr3 and Signr5, and determined their phenotype in TB infection (Tanne et al., 2009). The mycobacterial load in the lungs of Signr3-deficient mice was clearly increased, whereas knockout mice for Signr1 and Signr5 were indistinguishable from wild-type controls. However, the development of adaptive immune response to TB was not compromised in the absence of SIGNR3 and the mice did not die at higher rates compared to controls of TB. Of note, SIGNR3 was up-regulated on lung phagocytic cells during infection, and the increased mycobacterial load was specific for the lung while no difference between WT and Signr3$^{-/-}$ mice was seen in the spleen. SIGNR3 binding of ManLAM and whole mycobacteria induces production of proinflammatory cytokines through activation of Syk and the kinase Raf1 (Tanne et al., 2009). Although the requirement for Card9 in the activation of macrophages and DC in response to ligation of SIGNR3

has not been formally demonstrated, it appears very likely. Hence, a defect in the signaling of SIGRNR3 may contribute to the strong phenotype of the Card9$^{-/-}$ mice in tuberculosis, although it appears to be essential for anti-mycobacterial resistance only during the early innate phase of infection.

Given the large number of CLR that can be expressed in myeloid cells, additional players must be expected to contribute to recognition of mycobacterial ligands and to macrophage activation and anti-mycobacterial defense via Card9-dependent mechanisms. Another interesting candidate to look at is Dectin-2; of note a Dectin-2-Fc fusion protein binds to the surface of mycobacteria (McGreal et al., 2006). Similar to Mincle, Dectin-2 utilizes the FcRγ chain as adapter molecule. Interestingly, FcRγ chain-deficient mice develop increased mycobacterial burden, enhanced immunpathology and earlier death when challenged with MTB (Maglione et al., 2008). While Chan and colleagues discuss their findings exclusively in the context of FcRγ chain's role as adapter of activating Fc gamma receptors mediating antibody effector function, the dual use of the adapter in recruiting Syk also to several CLR family members could also indicate that the phenotype is at least in part due to a lack of signaling by Mincle, Dectin-2 and other CLRs. Likewise, the activation of the FcRγ-Syk-Card9 signaling cascade by Fc gamma receptors would allow a contribution of antibody-mediated effects to Card9-dependent protection in TB infection, a possibility that has not been explored to date.

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Modeling the *Mycobacterium tuberculosis* granuloma – the critical battlefield in host immunity and disease

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Granulomas are the hallmark of *Mycobacterium tuberculosis* (*M.tb*) infection and thus sit at the center of tuberculosis (TB) immunopathogenesis. TB can result from either early progression of a primary granuloma during the infection process or reactivation of an established granuloma in a latently infected person. Granulomas are compact, organized aggregates of immune cells consisting of blood-derived infected and uninfected macrophages, foamy macrophages, epithelioid cells (uniquely differentiated macrophages), and multinucleated giant cells (Langerhans cells) surrounded by a ring of lymphocytes. The granuloma's main function is to localize and contain *M.tb* while concentrating the immune response to a limited area. However, complete eradication does not occur since *M.tb* has its own strategies to persist within the granuloma and to reactivate and escape under certain conditions. Thus *M.tb*-containing granulomas represent a unique battlefield for dictating both the host immune and bacterial response. The architecture, composition, function, and maintenance of granulomas are key aspects to study since they are expected to have a profound influence on *M.tb* physiology in this niche. Granulomas are not only present in mycobacterial infections; they can be found in many other infectious and non-infectious diseases and play a crucial role in immunity and disease. Here we review the models currently available to study the granulomatous response to *M.tb*.

Keywords: *Mycobacterium tuberculosis*, model, granuloma, tuberculosis, pathogenesis

INTRODUCTION

An estimated one-third of the world's population carries an asymptomatic infection with *Mycobacterium tuberculosis* (*M.tb*), which results in eight million new cases of tuberculosis (TB) and two million deaths every year (WHO, 2011). Granulomas are the hallmark of *M.tb* infection and thus sit at the center of TB immunopathogenesis. TB can result from either early progression of a primary granuloma during the infection process (rare) or reactivation of an established granuloma in a latently infected person (10% lifetime risk in an otherwise healthy individual).

Granulomas are well-organized, dynamic structures with immune cells at various stages of differentiation (**Figure 1**). The cellular composition of TB granulomatous lesions includes blood-derived infected and uninfected macrophages, foamy macrophages, epithelioid cells (uniquely differentiated macrophages), and multinucleated giant cells (Langerhans cells), B and T lymphocytes, and fibroblasts (Russell, 2007; Ramakrishnan, 2012).

In human pulmonary TB, the granuloma formation process starts shortly after infection. When inhaled, *M.tb* is ingested by and transported across the alveolar epithelium by AMs into the lung tissue and adjacent lymph nodes, and then dissemination ensues through the lymphatics and blood stream. This process initiates a cascade of events involving the production of pro and anti-inflammatory cytokines and chemokines. The immune response generated stimulates the activation of phagocyte antimicrobial activities and leads to the recruitment of additional mononuclear leukocytes into the site of infection. This accumulation of cells around the foci of infected cells leads to the formation of a macrophage-rich cell mass known as a granuloma.

Mycobacterium tuberculosis can persist for decades within the granuloma structures and, due to some intervening medical (e.g., HIV infection, diabetes, cancer, malnutrition, aging, etc.) and/or genetic factors, bacteria can reactivate. A balance of pro-inflammatory and anti-inflammatory immune responses is essential for controlling bacterial proliferation within granulomas and the resolution of these granuloma lesions over time. A dysregulation in the immune response will lead to granuloma progression and disease. An accumulation of caseum in the center of the granuloma promotes an increase in necrotic tissue and the collapse of the granuloma center which releases virulent bacilli to other parts of the tissue where more lesions will be formed. In the lungs, breakdown of the granuloma into the airways can lead to transmission of bacteria to other individuals.

In general, the production of chemokines is essential for the recruitment of inflammatory cells to the site of infection (Algood et al., 2003). Particularly, the chemokines binding to the CCR2 receptor (CCL2/MCP-1, CCL12, and CCL13) play an essential role for the early recruitment of macrophages. During the early stages of granuloma formation, TNF produced by infected macrophages

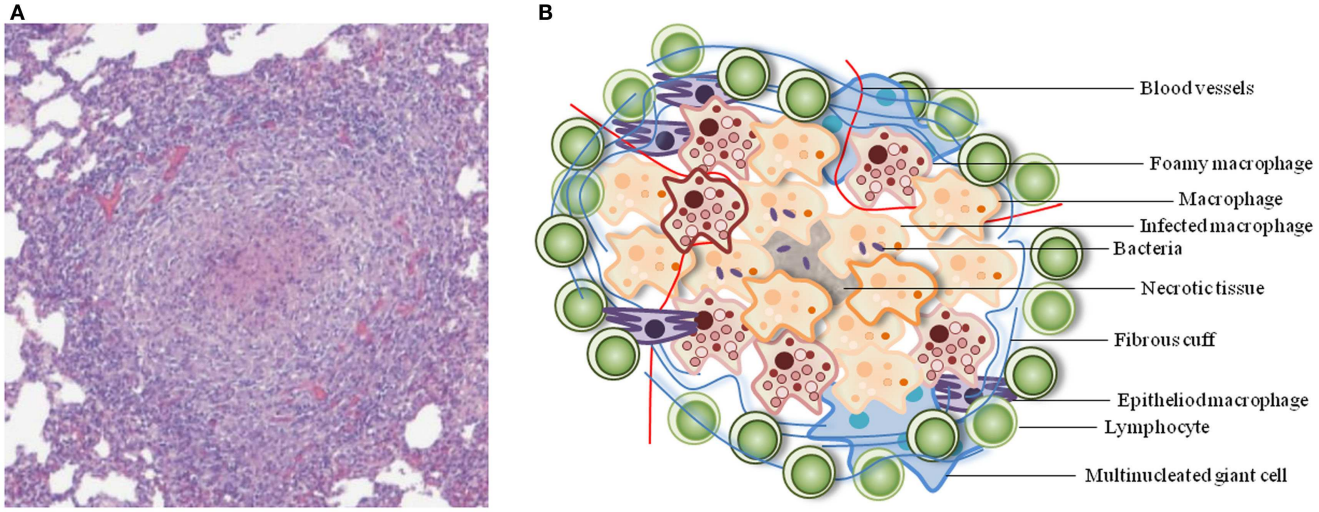

FIGURE 1 | Typical architecture of a TB granuloma. (A) Representative granuloma with central necrosis from minipig lung tissue. Histological samples were formalin-fixed, cut, and stained with hematoxylin-eosin. Adapted from Gil et al. (2010). **(B)** Schematic of the cellular constituents of a TB granuloma.

and T cells plays a crucial role in maintaining the granuloma structure by keeping sustained levels of chemokines and cellular recruitment and retention (Roach et al., 2002; Chakravarty et al., 2008). The accumulation of infected antigen presenting cells (macrophages and dendritic cells) in the regional lymph nodes leads to the development of the adaptive immune response against *M.tb*. Briefly, a type 1 T helper (T_H1) immune response is generated and CD4 T cells secrete IFNγ, IL-2, and lymphotoxin A. Additional types of T cells also contribute to the immune response against *M.tb* infection, including CD8 T cells and γδ T cells. However, granulomas develop during experimental infection in mice deficient in these cells (D'Souza et al., 2000; Mogues et al., 2001). CD8 T cells seem to be more important, at least in the mouse model, at later stages by producing IFNγ and inducing cytotoxic activity (Lazarevic and Flynn, 2002) once the bacillary growth is stable. It has been proposed that while the early stages of infection are marked by M1 macrophage polarization, mostly due to IFNγ secretion, providing the macrophages their mycobactericidal capacity, the later stages promote a shift toward M2 polarization as a consequence of several factors, including PPARγ and STAT6 expression (Lugo-Villarino et al., 2012). This scenario would be predicted to contribute to the formation of foamy macrophages (CD36 expression) and giant cells. Although granuloma formation has long been thought to be a host-driven process, more studies indicate that there is an active role played by *M.tb* (Davis and Ramakrishnan, 2009).

Several studies have been unraveling the role of the recently identified Th17 immune response within *M.tb* granulomas. The γδ T cell population is a major source of early IL-17 during mycobacterial infections (Lockhart et al., 2006; Umemura et al., 2007), especially upon high dose infection (Hamada et al., 2008). IL-17 is most commonly associated with a pro-inflammatory response and it has been suggested to play a role during early stages of the granuloma formation promoting PMN recruitment and organization around the foci of infection (Seiler et al., 2003; Torrado and Cooper, 2010). Neutrophils and macrophages cooperate for efficient mycobacterial killing (Silva, 2010).

During the aging process there are alterations in the immune system that affect T cell functions such as decreased cytokine production (IL-2 and IFNγ), cytotoxic activity, and T cell proliferation. Animal models, more specifically the mouse model, have clearly documented the relationship between age-related decreased T cell responses and the increased risk of infection by *M.tb*, as well as the negative impact of dysregulated immune responses during *M.tb* chronic infection (Turner et al., 2001a,b; Turner and Orme, 2002). Other factors such as other diseases (e.g., diabetes mellitus), poor nutrition, and immunosuppression also impact the protective granulomatous response against *M.tb* infection during aging (Yoshikawa, 1992).

Histologically, there are different types of granulomas. Initially, epithelioid cells may be surrounded by an acellular necrotic region, with a ring of B and T cells. The granulomas can displace parenchymal tissue and may necrotize, caseate, and/or calcify. Caseous granulomas might turn calcified during chronic or latent infection. Other types of granulomas may not have a necrotic area and are composed primarily of macrophages and a few lymphocytes. Host-pathogen interactions in the granuloma over the course of infection lead to adaptive changes of the tubercle bacilli, phenotypes of the host immune cells, and levels of the immune mediators they produce. These features allow for the formation of a wide spectrum of granuloma structures even within a single human host, therefore implying the presence of several unique microenvironments for *M.tb* as well as for the immune response.

On the one hand, the granuloma's main function is to localize and contain *M.tb* while concentrating the immune response to a limited area. On the other hand, complete eradication does not occur since *M.tb* has its own strategies to persist within the granuloma and to reactivate and escape under certain conditions.

Thus *M.tb*-containing granulomas represent a unique battlefield for dictating both the host immune and bacterial response. Bacterial dissemination outside of the lungs that normally follows primary infection allows for granuloma formation in many tissue sites throughout the body, again representing different immune microenvironments. The immune microenvironment which is highly dependent on the tissue location and associated cells, growth factors, and cytokines present will determine the pattern of differentiation of the cells forming granulomas, especially the macrophages. If the antigen load at the initial site of infection and regional lymph node is large, necrosis and caseum develop which represent signatures of *M.tb* granulomas. Reactivation TB occurs in the lungs 80% of the time, whereas in 20% of cases, TB reactivates at other tissue sites (e.g., pleural space, lymph nodes, bone, kidney, etc) (Frieden et al., 2003), a consequence of the early dissemination process that occurs during primary infection. Tuberculous lymphadenitis is one of the most common forms of all extrapulmonary TB.

Differentiating the protective mechanisms involved in the mycobacterial granulomatous response from those causing tissue damage and *M.tb* spread is crucial for the TB field. The architecture, composition, function, and maintenance of granulomas are key aspects to study since they are expected to have a profound influence on *M.tb* physiology in this niche. Current knowledge about the development and maintenance of granulomas in TB is limited. However, several approaches are being pursued recently to gain more insight into the events occurring within TB granulomas. Here we review the models currently available to study the granulomatous response to *M.tb*.

MODELING THE *M.tb* GRANULOMA

Use of *models* to study *M.tb* granulomas that resemble those in humans is necessary because: (1) it is difficult to study human lung biopsy samples since their access is often limited; (2) human biopsy samples provide a static image that needs to be extrapolated in order to understand the dynamic processes that take place within the granuloma; and (3) *M.tb* lacks a natural host beyond humans and, therefore, surrogate models are necessary that more or less resemble human granulomas. These models are classified as *in vivo*, *in vitro*, and *in silico* models (See **Table 1**).

IN VIVO MODELS

Animal models are often used to study the TB granulomatous response. These models reproduce several of the processes occurring in humans, although important differences are frequently observed.

Mice

The mouse is the most popular animal model of *M.tb* infection. The advantages of this model include size, availability and cost, the abundant immunological tools and reagents available, and the potential for manipulation, including use of inbred, genetically modified strains (Orme, 2003). The large number of mouse models generated for infection has contributed to our understanding of the granulomatous response to *M.tb*. The most relevant is the low inoculum aerosol infection model because the aerosol route mimics the natural route of infection in humans.

Resistant strain C57BL/6 mice infected intravenously or via aerosol with moderate or low-dose inocula, respectively, develop a chronic, progressive infection with *M.tb* (North and Jung, 2004; Orme, 2005; Basaraba, 2008), with the presence of non-replicating bacilli (Rees and Hart, 1961; Wallace, 1961; Munoz-Elias et al., 2005). By 21 days post infection, a mycobacterial burden plateau is reached ($\sim 10^5$–10^7 CFU) followed by a steady state of infection maintained for more than 1 year (Rhoades et al., 1997). However, despite the mice surviving the infection for a long time, where the bacillary load is initially controlled due to a strong T_H1 immune response, progressive infiltration of the lung and other tissues occurs due to changes in innate and adaptive immunity with age toward a more T_H0–T_H2 immune response (Cardona et al., 2000, 2003) and eventually all of the mice die from TB. This latter observation has led many to question the utility of the standard mouse model for studying granulomas in latency (Flynn, 2006).

The second mouse model widely used to study latent TB infection (LTBI) is the Cornell model. It is characterized by a high intravenous injection of $1–3 \times 10^6$ virulent *M.tb* followed by isoniazid and pyrazinamide treatment for 20 weeks (McCune and Tompsett, 1956; McCune et al., 1956). Despite obtaining non-culturable *M.tb* after treatment, mice are capable of developing spontaneous or induced reactivation after 90 days (McCune et al., 1966). The lack of a standardized protocol for establishing latency with the Cornell model and the lack of stability are significant disadvantages. Factors that can alter the outcome are the genetic background of the mouse strain used, strain of *M.tb* used, inoculum preparation protocol, route of infection, and the time between infection and treatment (Scanga et al., 1999).

Given the relatively high bacterial load and progressive pathology, these two models are recognized better for resembling a chronic infection, rather than a human latent infection. However, the features of low-dose aerosol infection, healthy appearing infected mice, and episodes of reactivation are generally consistent with human LTBI. Another limitation of the standard mouse model is the lack of structure and organization of the granulomas formed which do not resemble human granulomas. Granulomas in most mouse strains are formed by loose non-necrotic cellular aggregates, a discrete fibrotic reaction, lack of encapsulation, and a strong lymphocyte presence (Rhoades et al., 1997).

Recently, new models have been developed where granulomas develop necrotic lesions in response to *M.tb* infection and thereby resemble granulomas in humans more closely (Pichugin et al., 2009; Reece et al., 2010; Driver et al., 2012; Harper et al., 2012). Intravital microscopy studies have revealed the dynamic nature of mouse TB granulomas through three-dimensional time-lapse microscopy which show activated T cells entering and moving throughout the granuloma among relatively fixed macrophages (Egen et al., 2008, 2011). A relatively new approach to study cell traffic, repopulation, and the relationship between systemic immunity and mycobacteria-containing granulomas is the granuloma transplantation model (Harding et al., 2011). In this model, a mouse liver infected with BCG or *M.tb* is transplanted by surgical insertion underneath the recipient's kidney capsule. Interesting, new insight is being provided by this model. However, the surgical procedure, immune reaction to physical stress, length of survival

Table 1 | Models to study *M.tb* granulomas.

Models	Advantages	Disadvantages
Mice*	Inexpensive, easy to handle, genetic variant strains, large number of immunological tools, and reagents available	Lack of necrosis, lack of cell structure and organization that resemble human granulomas; lack of true latency
Guinea pig/rabbit*	Easy to handle, necrosis	Limited availability of immunological tools; lack of true latency
Non-human primate*	Lesions similar to human, LTBI established	Difficult to handle, dedicated veterinarian staff required, expensive, ethical concerns
Minipig*	Pulmonary structure similar to humans, LTBI established, lesions similar to humans	Difficult to handle, dedicated veterinarian staff required, expensive, limited availability of immunological tools
Zebrafish embryo*	Easy to handle, live, real time imaging, excellent to study initial steps of granuloma formation	*M. marinum* (surrogate bacterium), lack of lung structure, lack of lymphocytes
In vitro human	Mimics human granuloma structure, flexible (mycobacterial strains, manipulate with, e.g., cytokines, drugs), amenable to manipulation experimentally, use for drug screening	Lack of lung structure and full tissue microenvironmental conditions
In silico	Inexpensive, flexible, long-term experiments with multiple, complex factors can be quickly performed; hypothesis-generating	Highly dependent on the parameters chosen, requires previous observations in different systems to extrapolate, can miss unknown factors, often not tested or proven

**In vivo models.*

of transplanted tissue, and difficulty in applying this approach to other animal species are major limitations of this model.

Guinea pig/rabbit

In Guinea pigs and rabbits, some granulomas are more human-like and studies in these species have yielded important insights on the development and structure of granulomas (Flynn, 2006).

Guinea pigs are highly susceptible to low-dose aerosol infection and therefore do not establish a LTBI. Rabbits, despite being relatively more resistant to *M.tb* infection, also succumb to the infection. In both models the histopathology of granulomatous lesions is similar to that seen in progressive human disease as a strong inflammatory response with fibrosis and intragranulomatous necrosis followed by mineralization or even softening and liquefaction is seen (Lenaerts et al., 2007). A down side of these models is the relative lack of immunological tools and reagents available.

Non-human primate

The first evidence for progression from LTBI to active disease came from the Cynomolgus macaque, a non-human primate (NHP) model (Capuano et al., 2003). NHPs, including rhesus monkeys and macaques, have been used as models for *M.tb* infection (Flynn et al., 2003). NHPs develop a disease similar to that in humans presenting a wide spectrum of human-like lesions and varied outcomes to infection. In fact, NHPs are an excellent model to study the pathogenesis and immunology of TB, as well as to screen new vaccines, diagnostic reagents, and drug treatments for pre-clinical studies. Some disadvantages of this model include cost, difficulty with handling the animals, and ethical considerations.

Minipig

Recently, Gil et al. have described a model of TB infection in minipigs (Gil et al., 2010). In this model, the most characteristic feature is a strong local granulomatous response that is based on the induction of a fibrotic process, where lesions are encapsulated, and intragranulomatous necrosis and calcification are present to help contain the dissemination of bacilli toward the alveolar space. The initial lesions in this model show a mixture of neutrophils, macrophages, and lymphocytes without much organization, and very few bacilli. Over time, there is an increase in fibroblast proliferation, which leads to an accumulation of myofibroblasts, and the formation of a capsule around the granuloma, which appears to be critical in preventing the spread of bacilli. The parenchyma structure of the lung, characterized by extensive interlobular and intralobular connective tissue, may play a role in the evolution of LTBI. The fact that the local pulmonary structure in minipigs is similar to that in humans makes it a viable model to study the genesis of LTBI and the granulomatous response. This model is infrequently used in the field.

Zebrafish embryo

Infection of zebrafish embryos with *Mycobacterium marinum*, an aquatic, close genetic relative of *M.tb*, develops organized, necrotic granulomas which appear to recapitulate human caseous granulomas. Importantly, the optical transparency of the zebrafish embryo has provided a unique tool for visualizing the dynamics of primary granuloma formation and dissemination to generate new secondary granulomas during the innate immunity phase of the infection in real time (Davis et al., 2002; Rubin, 2009). Although using *M. marinum* instead of *M.tb* is a disadvantage of the model, it provides the advantage of modeling TB in its natural host. On the other hand, the lack of lymphocytes and lung structure are limiting factors of this model.

IN VITRO MODELS

Several reports have described models of *in vitro* granuloma formation using peripheral blood mononuclear cells (Franklin et al.,

1995; Seitzer and Gerdes, 2003; Birkness et al., 2007) involving collagen matrix gels and agarose beads or agarose-coated plates. Puissegur et al. (2004) described an *in vitro* granuloma model based on human peripheral blood mononuclear cell cultures either treated with mycobacterial antigen-coated beads or infected with mycobacteria. This model demonstrates the progressive recruitment of macrophages around live bacilli or mycobacterial antigen-coated beads, differentiation of these macrophages into multinucleated giant cells and epithelioid cells and, finally, recruitment of a ring of activated lymphocytes surrounding the granuloma structure. The epithelioid cells generated in this model have morphological and differentiation characteristics similar to those found in natural granulomas (Lay et al., 2007). This model has increased our knowledge about cell differentiation, cellular interactions and cell/bacteria interplay within the granuloma structures (Peyron et al., 2008; Russell et al., 2009). These models can be used to study the initial steps of granuloma formation and maintenance, and have the potential to address more translational aspects of human *M.tb* infection. On the other hand, they lack lung structure and thus the full tissue microenvironmental condition.

IN SILICO MODELS

In silico experimentation refers to research conducted via computer simulations with models and tools that are applied to generate new hypotheses and knowledge about biological systems. As is the case for any model, the *in silico* models are systems which are applied to a specific situation, and attempt to simplify a very complex system that cannot otherwise be adequately interrogated experimentally for investigation. Computer models have been developed that describe or predict the granulomatous response outcome based on previous experimental observations and general information about TB disease (Segovia-Juarez et al., 2004; Fallahi-Sichani et al., 2011; Marino et al., 2011). These models are inexpensive, very flexible, and incorporate a number of complex parameters. They are able to ask questions that cannot be easily investigated in the laboratory and generate new hypotheses. However, they are highly dependent on the parameters chosen, require previous observations in different systems to extrapolate the results and can miss unknown factors.

MODELING OTHER GRANULOMATOUS DISEASES

Granulomas usually serve to protect the host from the spread of persistent microorganisms or other enduring injurious substances. Therefore, they are not restricted to *M.tb* infection; there are other bacterial, fungal, and viral infections, and even non-infectious inflammatory diseases characterized by the presence of granulomas (Sandor et al., 2003). Some of the better studied granulomatous responses relate to other infectious diseases, neoplastic processes, and autoimmune inflammatory diseases. Below is a brief summary of some of them and their main characteristics:

- Leprosy: caused by *Mycobacterium leprae* and characterized by the presence of acid-fast bacilli within macrophages in granulomas. The most commonly used models are mice, armadillos, and mathematical models (Scollard et al., 2006; Adams et al., 2012).
- Syphillis: caused by *Treponema pallidum* and characterized by the presence of gumma, a microscopic to grossly visible lesion, enclosing a wall of histiocytes, with plasma cell infiltrates and central necrosis without loss of cellular outline. The most commonly used models are hamster and mathematical models (Kajdacsy-Balla et al., 1993; Gesink Law et al., 2006).
- Sarcoidosis: caused by an unknown etiology (although mycobacterial antigens have long been implicated) and characterized by non-caseating granulomas with abundant activated macrophages. The most commonly used model is the murine model of antigen-driven granuloma formation (Samokhin et al., 2011; Yeager et al., 2012).
- Crohn's disease: caused by immune reaction against intestinal bacteria and/or self-antigens and characterized by non-caseating granulomas in the wall of the intestine, with dense chronic inflammatory infiltrate. The most commonly used model is the murine model of chronic inflammation (Dillman et al., 2013; Tlaxca et al., 2013).
- Schistosomiasis: caused by *Schistosoma* ssp. and characterized by hepatic granuloma formation with fibrosis initiated by MHC-II-dependent, α/β^+ $CD4^+$ T lymphocytes. The most commonly used model is the SEA (*Schistosoma* egg antigen)-specific driven granuloma in mouse, monkey, and baboon animal models (Stavitsky, 2004).

Despite the extreme diversity in their etiology, several of the above diseases share general underlying histopathologic characteristics of granuloma lesions. Thus, the knowledge obtained about mycobacterial granulomas and some of the models used to study them may be useful in advancing knowledge about these other diseases.

CONCLUSION

This review has described a variety of experimental models available that can help decipher the complex host-pathogen relationship that takes place within the tuberculous granuloma. Although the mycobacterial granuloma seems to be a host defense mechanism for walling off *M.tb*, the bacilli can also survive, protected from killing by immune cells, and persist in a latent form until an opportunity arises for reactivation and dissemination (Grosset, 2003). An understanding of the pathophysiology of granulomas is critical for the design of new TB drugs and vaccines. Different models are necessary to cover the wide histopathological spectrum of mycobacterial granulomas observed. Each model described in this review has and, by further development, will continue to make important contributions to TB research. The luxury of having many available models, however, must be weighed against a careful interpretation of the data obtained from them. Given the advantages and disadvantages of each model, it seems most likely that our understanding of the mycobacterial granuloma will be derived from a combination of models. Granulomas are not only present in mycobacterial infections, they can be found in many other bacterial, fungal, parasitic, or viral infections, and even in non-infectious, inflammatory granulomatous diseases (Sandor et al., 2003). Therefore, the knowledge obtained from analyzing models

of mycobacterial granulomas may prove to be beneficial for uncovering mechanisms for other granulomatous diseases, including investigation of distinct infectious disease phenotypes and autoimmune as well as auto-inflammatory granulomatous diseases (e.g., Crohn's disease or sarcoidosis).

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Functional complexity of the *Leishmania* granuloma and the potential of *in silico* modeling

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In human and canine visceral leishmaniasis and in various experimental models of this disease, host resistance is strongly linked to efficient granuloma development. However, it is unknown exactly how the granuloma microenvironment executes an effective antileishmanial response. Recent studies, including using advanced imaging techniques, have improved our understanding of granuloma biology at the cellular level, highlighting heterogeneity in granuloma development and function, and hinting at complex cellular, temporal, and spatial dynamics. In this mini-review, we discuss the factors involved in the formation and function of *Leishmania donovani*-induced hepatic granulomas, as well as their importance in protecting against inflammation-associated tissue damage and the generation of immunity to rechallenge. Finally, we discuss the role that computational, agent-based models may play in answering outstanding questions within the field.

Keywords: granuloma, leishmaniasis, visceral, inflammation, *in silico* modeling, imaging

INTRODUCTION

Visceral leishmaniasis (VL), a parasitic disease impacting on health and economy in developing countries, is caused by *Leishmania donovani* and *L. infantum*. These parasites establish long-term infection within multiple organs including the spleen, liver, and bone marrow. In humans and dogs, VL is invariably fatal if untreated, but subclinical infections are common and are associated with granuloma formation (Pearson and Sousa, 1996; Sanchez et al., 2004). In experimental VL (EVL) in mice, granuloma formation is associated with self-limiting hepatic infection, whereas granulomas fail to form in spleen, where parasites persist (Murray, 2001). Together, these observations suggest a causal association between granuloma formation and host resistance to visceralizing species of *Leishmania*.

Granulomas progress through distinct stages of "maturation," as described in **Figure 1**. Fundamental insights into the role of different immune cells, effector and regulatory cytokines, and other mediators have been made through the use of gene-targeted mice (reviewed in Murray, 2001; Kaye et al., 2004; Stanley and Engwerda, 2007). However, the approach of using knockout (KO) mice or blocking/depleting antibodies is limited when asking questions about immune regulation within these discrete inflammatory foci. In this mini-review, we will attempt to link recent studies involving direct visualization of hepatic granulomas with previous findings, discuss how the granuloma may function at a cellular and spatiotemporal level and highlight important unanswered questions. We also discuss the potential of *in silico* modeling to aid our understanding of these fascinating structures.

THE DYNAMIC MICROENVIRONMENT OF THE GRANULOMA

KC–NKT CELL INTERACTIONS AND T CELL RECRUITMENT

In EVL, Kupffer cells (KCs) are at the heart of the hepatic granuloma, often fusing with each other as a result of migration from neighboring sinusoids (Murray et al., 1987; Beattie et al., 2010a). The signals that trigger KC migration remain to be identified. The KC-rich core acts as a platform for the recruitment of other cells, notably T cells and monocytes (Beattie et al., 2010a). The exact mechanisms resulting in cell recruitment are unknown, although blood flow, adhesion molecules (Murray, 2000; Engwerda et al., 2004), chemokines (Sato et al., 1999), and cytokines such as interleukin (IL)-1 (Curry and Kaye, 1992) have all been implicated.

Invariant natural killer T cells (iNKTs) play a significant amplifying role. iNKTs-deficient mice have impaired granuloma maturation, lower inflammatory cytokine expression, and reduced expression of CCL2, CXCL5, and CXCL2 (Robert-Gangneux et al., 2012). iNKTs interact with KCs via a signal regulatory protein alpha-CD47 dependent amplification loop to regulate the production of the T cell-chemoattractant CXCL10 (Svensson et al., 2005; Beattie et al., 2010b), shown to be host protective in EVL (Gupta et al., 2009). KC–NKT interactions also feature in other infection models (Lee et al., 2010), but the consequences of NKT cell activation may not always be favorable. For example, treatment with α-galactosylceramide, an activator of iNKTs, decreased rather than enhanced resistance (Stanley et al., 2008). It remains to be directly shown whether iNKTs are retained within granulomas but $cxcr6^{gfp/+}$ mice (Geissmann et al., 2005) could be used to address this question.

T CELLS: STRENGTH IN NUMBERS?

T cells are the predominant cell type present within the *L. donovani*-induced granuloma and granuloma maturation (**Figure 1**). Both $CD4^+$ and $CD8^+$ T cells are indispensable in the effective formation and function of granulomas and for parasite clearance (Stern et al., 1988). 4D intravital imaging studies in EVL and in mice with bacille Calmette–Guérin (BCG) infection

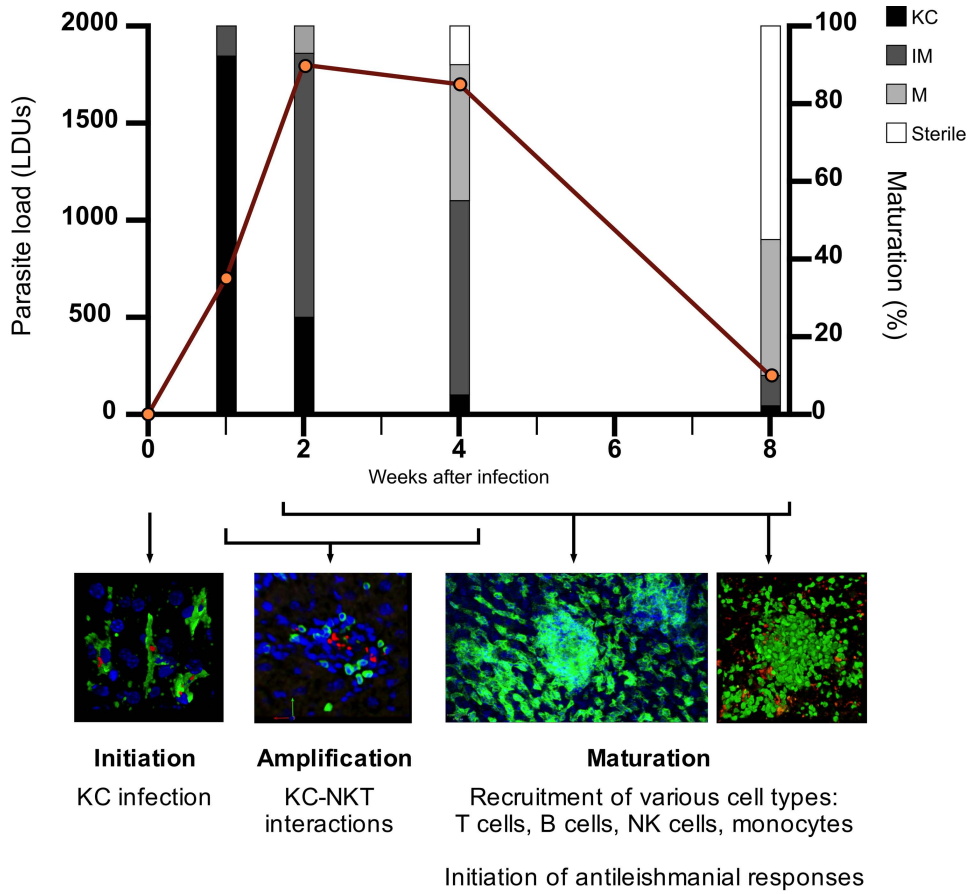

FIGURE 1 | The process of granuloma maturation during *L. donovani* infection. The infection of resident liver macrophages (Kupffer cells; KCs) initiates the process of granulomatous inflammation (*initiation*). Hepatic NKTs migrate toward infected KCs and their interaction triggers the recruitment of mononuclear cells to the liver (*amplification*). Various cell types, predominantly T cells, are recruited to infected KC foci within the liver, with granulomas developing in size and cellularity in an asynchronous manner over the first 4 weeks post-infection (p.i.) (*maturation*). During the *maturation* stage, the inflammatory response peaks around 4 weeks p.i. where the antileishmanial response becomes sufficient to begin clearing the parasite burden. As parasites are cleared from individual infected KC foci, cells begin to move out of the granuloma, returning the liver to its original pre-infection state. Hepatic granulomatous inflammation resolves by 8 weeks p.i. with the majority of parasites cleared. Data on parasite load and granuloma maturation redrawn from Murray (2001). KC, Kupffer cell; IM, immature granuloma; M, mature granuloma; sterile, sterile granuloma.

indicate that T cells move relatively freely within granulomas, with no requirement for antigen-specificity for granuloma entry (Beattie et al., 2010a; Egen et al., 2011). Antigen presentation within granulomas appears limited in both these models. Of note, during *L. major* infection the effector function of a few antigen-specific $CD4^+$ T cells is enhanced by bystander activation (Muller et al., 2012). We have found that ∼70% of $CD4^+$ T cells within infected livers display an activated phenotype ($CD44^{hi}$), with ∼30–40% of $CD4^+$ T cells having the capacity to produce interferon-gamma (IFN-γ; manuscript submitted). These data are consistent with a model whereby local bystander activation operates within (and possibly even between) granulomas to enhance effector function. Future studies should aim to test such a model and determine whether non-specific T cell recruitment is beneficial for the outcome of *Leishmania* infection. Furthermore, the extent to which T cell functional differentiation occurs within the granuloma environment remains open.

THE ROLE OF GRANULOMA-ASSOCIATED MONOCYTES

Monocytes are present in *L. donovani*-induced granulomas but do not appear to be infected. In contrast, both infected and uninfected monocytes are recruited to BCG-induced hepatic granulomas (Egen et al., 2008). In a zebrafish embryo model, monocytes were recruited to *Mycobacterium avium*-induced granulomas throughout infection, and were shown to be capable of becoming infected, migrating away from the granuloma, and establishing infection in distant sites (Davis and Ramakrishnan, 2009). It is important to note that zebrafish embryos lack T cells (Willett et al., 1999; Trede et al., 2001) but, nonetheless, this suggests an interesting link between granuloma formation and disease dissemination. To date, we have no evidence that monocytes behave in this way during EVL. The blocking of monocyte recruitment to *L. donovani*-induced granulomas using anti-type 3 complement receptor (CR3) antibody resulted in impairment of early antileishmanial resistance followed by delayed granuloma maturation (Cervia et al., 1993). This indicates the importance of

monocytes in effective granuloma formation but we do not fully understand how the monocytes carry out this function. Similarly, it is unknown if a proportion of macrophages in the granuloma core represents differentiated migratory monocytes. Inflammatory monocytes are capable of migrating from the blood and promoting inflammation (Shi and Pamer, 2011). Thus, it would be informative to determine the phenotype of granuloma monocytes to best understand their ability to propagate the liver inflammatory response.

$CD11c^+$ dendritic cells (DCs) are also found within *L. donovani*-induced granulomas although they were shown not to be the targets of effector $CD8^+$ T cells (Beattie et al., 2010a). In addition, during BCG infection, $CD11c^+$ cells in chronic granulomas displayed decreased expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules whereas $CD11c^+$ cells within acute granulomas could support the reactivation of recruited antigen-specific $CD4^+$ T cells and could induce IFN-γ responses from naïve T cells (Schreiber et al., 2010). The role of DCs in antigen presentation to $CD4^+$ T cells in granulomas during EVL remains to be addressed.

BALANCE BETWEEN INFLAMMATION AND TISSUE DAMAGE

We have shown B cells to be recruited to granulomas throughout hepatic infection, and although antigen-specificity did not affect their recruitment, cognate B–T interactions could take place (Moore et al., 2012). We found no direct evidence of regulatory B cells, though these cells have been described by others in EVL (Deak et al., 2010), but nevertheless B cell-deficient mice have accelerated granuloma formation (with neutrophil infiltration) and enhanced parasite clearance during EVL (Smelt et al., 2000). B cells, therefore, play a role in preventing liver pathology, via the control of neutrophil infiltration, highlighting a divorce between the control of parasite burden and the induction of tissue pathology.

Liver pathology is also observed in the case of tumor necrosis factor (TNF) deficiency where high liver parasite burdens do not result in liver pathology until the severely delayed and exaggerated inflammatory response begins, resulting in hepatic necrosis (Murray et al., 2000). However, in contrast to other experimental models of hepatic inflammation and infection, such as schistosomiasis, EVL in wild type (WT) mice does not appear to induce overt liver pathology or fibrosis. The T helper type 1 (Th1)-dominated inflammatory response observed during EVL is not normally associated with fibrosis-initiating mechanisms. However, there is evidence suggesting hepatic fibrosis can occur in EVL, which is particularly relevant to human VL where more extensive fibrosis has been described (el Hag et al., 1994; Duarte et al., 2009). Hepatic fibrosis occurs in a range of chronic inflammatory conditions, and is typically triggered by the activation of hepatic stellate cells (HSCs) and the transition of stellate cells into myofibroblasts, resulting in deposition of collagen and other extracellular matrix proteins (Gressner and Bachem, 1995). Alternatively activated macrophages (AAM) are also implicated in the regulation of fibrosis (Wynn and Barron, 2010), and AAM-associated cytokines are implicated in the hepatic response during EVL. IL-13-deficiency affects granuloma maturation, but reports differ in the extent to which this affects parasite clearance (Murray et al., 2006; McFarlane et al., 2011). IL-4 KO and IL-4R KO mice also displayed delayed granuloma maturation, suggesting a role for IL-4 in the hepatic inflammatory response to *L. donovani* infection (Stager et al., 2003). Of interest, in mice co-infected with *L. donovani* and *S. mansoni*, hepatic granulomas fail to form around *L. donovani*-infected KCs in the vicinity of egg granulomas where AAMs are abundant, whereas *L. donovani*-infected KCs in the parenchyma do serve as a focus for granuloma maturation (Hassan et al., 2006). Further studies should aim to elucidate the function of HSCs and AAMs within the liver during EVL, and may help to create mouse models that are more representative of human disease.

The occurrence of overt pathology in various mouse KO models of EVL suggests that processes are present in WT mice to protect against inflammation-induced hepatic tissue damage. In addition to a regulatory role for B cells in EVL, natural killer (NK) cells have been shown to be present within *L. donovani*-induced granulomas and displayed immunoregulatory properties (Maroof et al., 2008). However, this study did not address whether the loss of NK cells led to enhanced liver pathology.

Whether regulatory T cells (Tregs) are recruited to *L. donovani*-induced granulomas and/or protect against pathology is unknown and is an important question to address. We have shown ∼2–3% of $CD4^+$ T cells within infected livers co-express IFN-γ and IL-10, indicative of a regulatory phenotype (manuscript submitted). Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is expressed by Tregs and is important for executing their immunosuppressive function (Read et al., 2000; Takahashi et al., 2000). We have shown that using anti-CTLA-4 blocking antibody during EVL enhanced granuloma maturation and parasite killing (Zubairi et al., 2004), one explanation for this result being that Tregs are operating to limit granulomatous inflammation. Whether this limitation protects against excessive inflammation and the development of tissue pathology is an interesting question to address. Granuloma-derived transforming growth factor-β (TGF-β) also inhibited IFN-γ production by $CD4^+$ T cells during EVL (Wilson et al., 1998), providing further evidence that regulatory mechanisms are operating with *Leishmania*-induced granulomas. Studies in schistosomiasis-associated granulomas demonstrated a role for regulatory CTLA-4^+ $CD25^-$ $CD4^+$ T cells in protection against host mediated pathology, with blocking of these Tregs resulting in significant weight loss but enhanced recruitment of effector T cells to hepatic granulomas (Walsh et al., 2007). Other studies have demonstrated the presence of Tregs within pulmonary granulomas, with Tregs being found in both *Mycobacterium tuberculosis* (*Mtb*) infection (Scott-Browne et al., 2007) and the autoimmune disorder sarcoidosis (Taflin et al., 2009). Understanding the balance between immunoregulation and inflammation-induced tissue damage in greater detail will prove crucial in optimizing future therapeutic approaches designed at enhancing the inflammatory response to infection.

Regulatory cells within *L. donovani*-induced granulomas may also function to control the resolution stage of hepatic granuloma formation. As describe in **Figure 1**, granuloma maturation peaks around 4 weeks post-infection (p.i.) followed by parasite clearance and the resolution of granulomas, enabling the liver architecture to

return to its pre-infection state. As the hepatic granuloma response continues to increase in the first 4 weeks of infection, it would be reasonable to posit that there must be a mechanism(s) in place to act as "a brake" on the immune response and begin the resolution stage of EVL. Whether the various cell types mentioned in the previous section play a role in this is an interesting topic that has not been previously addressed.

IMMUNITY

A final theme that has received little attention is whether or not granulomas are essential for the promotion and/or maintenance of memory required for immunity to reinfection. Secondary protection relied on $CD8^+$ T cells and resulted in accelerated granuloma development at infected foci (Murray et al., 1992). Interestingly, treatment with cyclosporin A during reinfection resulted in an absence of granuloma formation but did not affect protection (Murray et al., 1992). These data provides compelling evidence that granuloma formation may be dispensable in providing protection against *L. donovani* infection. However, to date, there has been no study of rechallenge that did not result in granuloma formation during the primary challenge. Thus, it is still unknown whether granuloma formation plays a role in generating memory responses and this question will be critical in addressing whether hepatic granulomas are indeed indispensable to immunity during EVL.

MODELING GRANULOMAS

THE POTENTIAL OF *IN SILICO* MODELING

Understanding the complex inner workings of the granuloma microenvironment is challenging. *Ex vivo* studies only provide experimental observations at individual snapshots in time and without blood perfusion, vital nutrients are lost and the liver sinusoidal structure and its contents become disrupted, leading to a rapid impairment of *ex vivo* liver function. Although manipulation of intact mice may alter granuloma form and function, such interventions also target events outside the granuloma microenvironment. *In silico* models can avoid these caveats and provide novel insight into systems through predictive modeling, providing time-course data and allowing an iterative process of hypothesis testing and experimental validation. This cycle of experimentation and validation can prove useful for ascertaining effects of experimental interventions.

Computational modeling of the dynamics of granuloma formation during *Mtb* infection (Segovia-Juarez et al., 2004; Gammack et al., 2005; Fallahi-Sichani et al., 2011) has helped elucidate the role of $CD8^+$ T cell effector function (Sud et al., 2006), DC trafficking and antigen presentation (Marino et al., 2004) and of TNF in granuloma maintenance (Ray et al., 2009; Fallahi-Sichani et al., 2010; Marino et al., 2010). These studies highlight the need to move from a more traditional reductionist study of biological systems, toward creating an "integrative picture of a system" using multi-scale modeling to capture system behaviors across both biological- and temporal-scales (Kirschner et al., 2007). Multi-scale modeling often integrates various modeling approaches, such as agent-based modeling (ABM) and equation-based modeling. In this review, we focus only on ABM.

AGENT-BASED MODELING OF GRANULOMAS IN THE LEISHMANIASES

Modeling granuloma formation in the leishmaniases is in its infancy, and our efforts have focused on ABM as a suitable technique for investigating granuloma formation, structure, and function. ABMs can be used to extend equation-based modeling approaches to account for spatiotemporal system dynamics (Germain et al., 2011). However ABMs can also be created and used as stand-alone simulation software. ABMs are typically built from behaviors and data derived from *in vivo* experimentation to explore hypothesized behaviors of cellular populations and cellular interactions.

Several studies have made use of ABM to study various forms of inflammation (Li et al., 2008; Dong et al., 2010; Brown et al., 2011), and a recent review has highlighted the benefits and challenges of the approach for inflammatory contexts (An and Christley, 2012). Indeed, ABMs have various advantages for studying inflammation and tissue-scale phenomena (**Figure 2A**). The study of granuloma formation is invariably linked to both the structure of the granuloma, and the spatial environment in which the granuloma is present. ABM allows for spatial representation of agents (e.g., cells, molecules), in addition to functional abilities/states for each agent (e.g., rate of cytokine production, receptor expression), providing the potential to compare biophysical and functional mechanisms. These models can represent agents individually with their own state providing heterogeneity within a population of agents, allowing for the study of small differences in individual agent behavior that manifest at the population level. Individual agent representation also provides the ability to interrogate and track agents. For example, this facilitates the study of cell recruitment into a granuloma, with respect to the surrounding environment, and the analysis of various interactions and environmental cues received by those cells, resulting in a powerful predictive tool (**Figure 2B**). The choice of ABM approaches should always match the purpose for the model. For example, modeling large populations of homogenous cells or molecules to study population level properties may lend itself more, in terms of efficiency, to mathematical approaches, although frameworks to efficiently model large homogenous populations with ABMs do exist (Holcombe et al., 2006).

Our initial *in silico* studies focused on developing tools to understand the well-documented heterogeneity of the granulomatous response that leads to fully formed mature granulomas residing side by side with infected KCs showing minimal inflammatory cell recruitment (**Figure 1**). This ABM provided a means for evaluating whether granuloma heterogeneity might reflect competition for NKT cells or KC diversity (Flugge et al., 2009), but was limited from a computational and biological perspective. A more complex stochastic Petri-net model investigated novel therapeutic interventions for host protection during *L. donovani* infection (manuscript submitted). This model suggests individual granulomas have a heterogeneous capacity for parasite clearance, and predicts that KC autocrine IL-10 is a key regulator of intra-granuloma effector mechanisms. Recently, we have developed a tissue-scale ABM to investigate the dynamics of NKT cell recruitment and KC stimulation during early infection, providing insight into interventions for the promotion of an early granulomatous response. Our model uses artificial sinusoidal

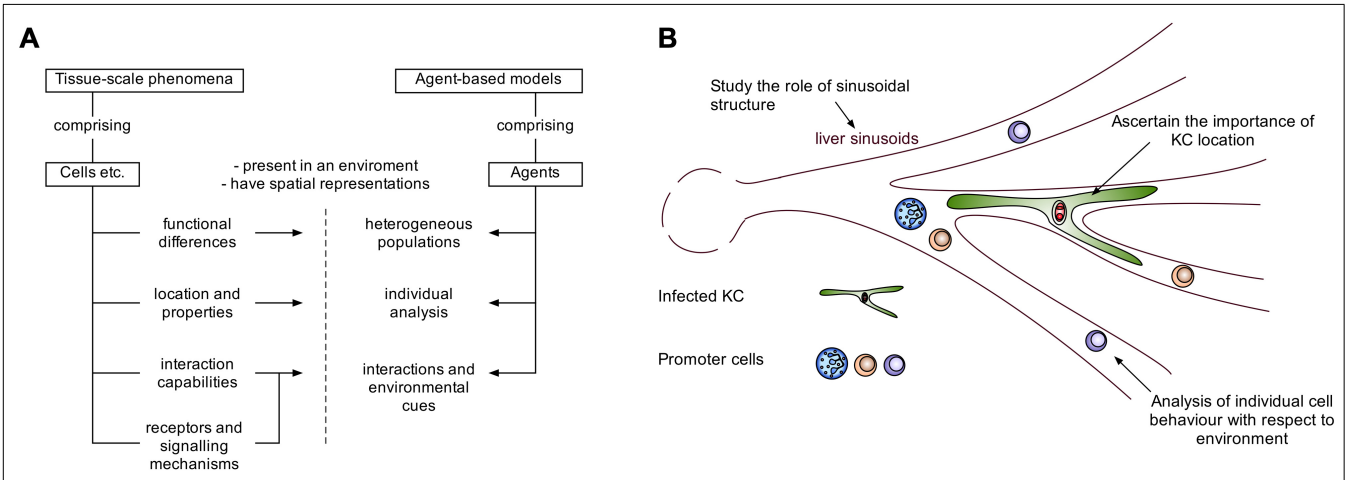

FIGURE 2 | The potential of agent-based modeling for modeling tissue-scale phenomena. ABMs allow the acquisition of time-course data with a custom degree of granularity. **(A)** A schematic demonstrating how features of entities such as cells in tissue-scale phenomena, can be captured by features of agent-based models. **(B)** Illustrating the advantages of agent-based modeling in the context of hepatic granuloma formation.

environments, generated using published imaging and statistical analysis data (Hoehme et al., 2010) and demonstrates the importance of liver architecture in granuloma positioning.

CONCLUSION

Leishmania donovani-induced granulomas represent an intriguing example of innate and adaptive immunity combining in a unique microenvironment to eradicate an intracellular pathogen. Each granuloma represents a highly organized structure and studies discussed in this review have shown that several different cell types and factors function within this complex structure to deliver effective parasite clearance, without causing excessive tissue damage. Interestingly, it is still unknown if granuloma formation is indispensable for immune protection but granulomatous inflammation likely provides the most efficient mechanism of delivering key antileishmanial processes in a focused manner. The advancement of imaging techniques has allowed us to study granulomas in greater detail, highlighting the dynamic and complex nature of these microenvironments, but as a result have raised important questions. The ability of ABMs to create an integrated system, incorporating the multiple factors involved in granuloma formation, offers a powerful tool to help address these questions, enabling the testing of hypotheses and discovery of potential interventions. Future studies should aim to use a combination of experimentation and modeling to unearth the complexities of the *Leishmania* granuloma and discover how exactly this response delivers its effective antileishmanial function.

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Chronic schistosome infection leads to modulation of granuloma formation and systemic immune suppression

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Schistosome worms have been infecting humans for millennia, but it is only in the last half century that we have begun to understand the complexities of this inter-relationship. As our sophistication about the inner workings of every aspect of the immune system has increased, it has also become obvious that schistosome infections have broad ranging effects on nearly all of the innate and adaptive immune response mechanisms. Selective pressures on both the worms and their hosts, has no doubt led to co-evolution of protective mechanisms, particularly those that favor granuloma formation around schistosome eggs and immune suppression during chronic infection. The immune modulatory effects that chronic schistosome infection and egg deposition elicit have been intensely studied, not only because of their major implications to public health issues, but also due to the emerging evidence that schistosome infection may protect humans from severe allergies and autoimmunity. Mouse models of schistosome infection have been extremely valuable for studying immune modulation and regulation, and in the discovery of novel aspects of immunity. A progression of immune reactions occurs during granuloma formation ranging from innate inflammation, to activation of each branch of adaptive immune response, and culminating in systemic immune suppression and granuloma fibrosis. Although molecular factors from schistosome eggs have been identified as mediators of immune modulation and suppressive functions of T and B cells, much work is still needed to define the mechanisms of the immune alteration and determine whether therapies for asthma or autoimmunity could be developed from these pathways.

Keywords: T helper lymphocytes, immune regulation, hygiene hypothesis, soluble egg antigen, sialyl Lewis^x glycans

INTRODUCTION

Schistosoma mansoni, Schistosoma haematobium, and *Schistosoma japonicum* are helminth worm species that infect humans and are highly prevalent in warm climates. They are obligate parasites that require a supply of blood from mammalian hosts to mature from larval stages to adult worms. Schistosomes live within the body of their hosts where they attach to the walls of intestinal blood vessels for feeding. Adult male and female schistosomes form copulating pairs that can produce as many as 300 eggs per couple per day. The eggs only hatch in fresh water, and the first larval form requires the presence of freshwater snails in order to mature into cercariae, the larval form that infects humans and other mammals. Unfortunately, a large portion of the eggs that are produced enter the portal circulation instead of leaving the body and deposit in internal organs, particularly the liver. As will be described in more detail later in this review, the immune system of the host recognizes schistosome eggs as foreign and responds with a local granulomatous response and systemic changes to immunity. The ability of adult schistosome worms to persist in their hosts and the resulting continual production of eggs and their antigens drives the adaptive immune system toward a highly regulatory response that has repercussions to overall immunity.

MODELING OF SCHISTOSOME EGG GRANULOMA FORMATION IN MICE

Most of what is known about the mechanisms by which schistosome granulomas form and function has come from the use of mouse models, especially in response to *Schistosoma mansoni* eggs (Boros, 1989). Schistosome cercariae can infect most if not all mammalian hosts and mice have proven very useful for studying granulomatous responses due to significant similarities with the human immune system, the availability of a vast array of reagents, and the production of many immunogenetically altered mouse strains that aid in mechanistic studies. The classic model used to study granuloma formation involves infection with schistosome cercariae, the larval form that infects humans, either through skin exposure or direct subcutaneous injection into the mouse. Adult worm pairs produce eggs continuously resulting in asynchronous granuloma formation. The natural infection model has been very useful in the study of the dynamics of the immune response, pathology, and granuloma architecture, but has some limitations due to its asynchronous nature. To study temporal aspects of egg deposition and granuloma formation, other models were developed in which purified schistosome eggs, or egg antigens coated to beads or macromolecular compounds were

injected into the tail veins of mice (Boros and Warren, 1971b). The intravenous injection model results in egg deposition primarily in the lungs, where the granulomas form simultaneously and temporal changes in the immune response can be more easily tracked. Differences have been noted between lung versus hepatic granulomas, and between the differing species of schistosome worms. Therefore, it is important to consider the route and form of administration, localization of granuloma formation, and the infectious agent when interpreting results.

THE SCHISTOSOME EGG GRANULOMA: A NECESSARY EVIL

Schistosome eggs have an outer shell made of chitin that houses the larval form, miracidiae, which is responsible for the release of soluble egg antigens (SEA). The miracidiae do not hatch in the host tissues, but production of SEA while the larvae are still viable stimulates the host immune response to form a granuloma (Boros and Warren, 1970). Using the temporal induction models, it was determined that the initial response to egg deposition and SEA release in small blood vessels involves the local production of inflammatory cytokines (TNFα, IL-1) and chemokines from resident epithelial cells and macrophages (Joseph and Boros, 1993; Lukacs et al., 1993; Wynn et al., 1993; Burke et al., 2010). This triggers the early influx of monocytes, neutrophils, and lymphocytes and the establishment of schistosome egg granulomas. Each schistosome egg and its individual granuloma do not pose much of a threat to the host. Over time, however, the constant deposition of eggs and formation of granulomas leads to hepatosplenomegaly and significant blockage of portal blood flow (Boros, 1989). Portal hypertension promotes the development of intestinal and esophageal varices resulting in severe bleeding and eventually can lead to the death of the infected individual. Schistosome egg granulomas were therefore viewed as major contributors to the pathogenesis of schistosome infection and strategies were sought to inhibit or prevent granuloma formation.

Several lines of evidence demonstrated that granuloma formation in response to SEA was dependent on the activation of $CD4^+$ T helper (T_H) lymphocytes and also highlighted the importance of granuloma formation to host survival. Infection of athymic nude mice led to substantially decreased granuloma size and impairment of the anti-SEA antibody response (Phillips et al., 1977; Amsden et al., 1980). Elimination of T_H cells by treatment with anti-lymphocyte serum or the specific, complement-fixing anti-CD4 antibody, L3T4, led to decreased granuloma formation and IL-2 production by spleen cells of infected mice (Domingo and Warren, 1968; Mathew and Boros, 1986). The severe absence of granuloma formation following high efficiency anti-CD4-depletion in mice led to the influx of low numbers of macrophages or eosinophils, diminished collagen deposition around eggs and increased damage to local hepatocytes (Mathew and Boros, 1986; Fallon et al., 2000b). Further evidence of the critical importance of T_H cell-mediated granuloma formation came from studies of infection in thymectomized mice and mice receiving additional T cell ablative therapies in which severe inhibition of granuloma formation led to mortality due to increased liver damage (Lucas et al., 1980; Fallon and Dunne, 1999). Toxicity to hepatocytes in the absence of granuloma formation has been attributed to the hepatotoxic cationic glycoproteins, α_1 and ω_1, released by schistosome eggs as part of SEA (Dunne et al., 1991; Abdulla et al., 2011). The mechanisms by which granulomas prevent direct hepatotoxicity have yet to be shown definitively, but of potential interest is a recent finding that the glycosylated T2 ribonuclease ω1 binds to the mannose receptor, a C-type lectin expressed by macrophages and dendritic cells (DC) (Dewals et al., 2010; Everts et al., 2012). Binding and internalization of ω1 through the mannose receptor on DC leads to polarization of these antigen presenting cells toward a T_H2 inducing phenotype (Everts et al., 2009, 2012). Therefore, infiltration of macrophages and DC into granulomas in the liver appears to be one host protective mechanism for binding and neutralizing hepatotoxic glycans from the schistosome egg and surrounding tissue and for the ultimate survival of the host. At the same time, the response to ω1 by DC and macrophages drives the T_H2 immune response leading to increased lymphocyte and eosinophil infiltration and enlargement of the granuloma (Everts et al., 2009; Steinfelder et al., 2009). As will be discussed below, this T_H2 dominated response may also be an adaptive mechanism of protection of the host from deleterious prolonged T_H1 mediated immunity. Chronic schistosome infection leads to further modulation of the immune response going from an acute T_H2 reaction toward immune regulation and fibrosis.

SCHISTOSOME GRANULOMA FORMATION AS A DYNAMIC MODEL OF ADAPTIVE IMMUNE RESPONSES

T_H1 CELLS MEDIATE EARLY GRANULOMA FORMATION

The role of T_H cell-derived cytokines in schistosome granuloma formation has been intensively studied and reviewed (Scott et al., 1989; Boros, 1994; Milner et al., 2010). A timeline of the changes in mouse T_H cell responses during the natural infection by schistosome cercariae is given in **Figure 1**. Following the initial proinflammatory cytokine release, $CD4^+$ T_H cells enter the lesion and release T_H1-type cytokines, IL-2 and IFNγ, which facilitates the establishment of delayed-type hypersensitivity response and early granuloma formation. A series of experiments led to the discovery of antigenic peptides, some that were able to induce T_H1-type immune responses even in the absence of an adjuvant (Lukacs and Boros, 1991, 1992, 1993; Cai et al., 1996; Chen and Boros, 1998, 2001; Reis et al., 2008). The finding that neutralization of IL-4 during schistosome infection led to decreased granuloma formation prompted interest in prolonging T_H1 responses during schistosome infection in order to reduce pathology (Yamashita and Boros, 1992). Initial results indicated that IL-12 could drive expression of IFNγ, IL-2, IL-10, and IL-12 in pulmonary granulomas and could reverse established T_H2-type responses in egg pre-sensitized animals (Wynn et al., 1994). Further studies indicated that vaccination of mice with eggs plus IL-12 led to decreased fibrosis similar to that seen in Stat-6 knockout mice that lacked IL-4 receptor signaling (Wynn et al., 1995; Kaplan et al., 1998). Much of the IL-12 adjuvant effect was attributed to induction of IFNγ (Boros and Whitfield, 1998). However, extreme skewing of the immune response toward schistosome eggs to a T_H1-type reaction resulted in increased mortality and liver pathology (Boros and Whitfield, 1998; Fallon and Dunne, 1999; Wynn and Hoffmann, 2000). In addition to the deleterious effects of an overt T_H1-type response in mice, it has also been

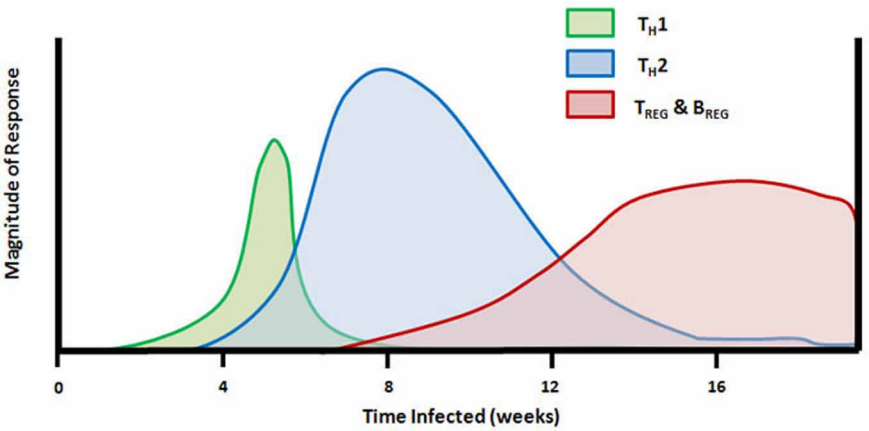

FIGURE 1 | Timeline of T_H cell mediated responses in the mouse model of schistosome infection. Subcutaneous injection of schistosome cercariae on day 0 leads to development of adult worms and egg production beginning at 4–5 weeks. The early innate and adaptive immune response to adult worm antigens is dominated by proinflammatory and T_H1 cytokines (TNFα, IL-12, and IFNγ). Following egg deposition in the liver and other internal organs, the larval miracidiae release soluble egg antigens (SEA) containing molecules that drive a rapid transition from T_H1 to T_H2-dominated immunity and production of IL-4, IL-5, and IL-13. Between 7 and 8 weeks of infection, $FoxP3^+$ T_{REG} cells and IL-10 are detectable, and the population of splenic $FasL^+$ $CD5^+$ B cells begins to proliferate. T_H2 response and peak granuloma formation occurs between 8 and 10 weeks of infection and is followed by granuloma downmodulation and increasing fibrosis around newly deposited eggs which persists throughout the remainder of infection.

shown that the more severe form of human disease, hepatosplenic schistosomiasis, is associated with elevated levels of TNFα and IFNγ and lower levels of the T_H2-type cytokine IL-5 (Mwatha et al., 1998). Thus, although decreased granuloma size and fibrosis in T_H1 dominated responses appeared to be desirable, it is clear that the switch to a T_H2-type response may have some protective effect for the host.

T_H2 CELLS AND NON-T CELL SOURCES OF IL-4

In the murine model, the cytokine response to worm eggs and SEA begins to switch to a T_H2-type response during the seventh week post-infection. By 8 weeks of infection, the production of IL-4 and other T_H2 cytokines predominates and IFNγ is barely detectable. The switch to a T_H2-type reaction is accompanied by a change in the cellularity of the granuloma with a dramatic influx of eosinophils, mast cells, and fibroblasts into the lesion. The influx of these cell types results in enlarged granulomas with increasing fibrosis. The mechanisms underlying this switch in cytokine pattern have been intensely studied. Neutralization of IL-4 or infection of IL-4-deficient or IL-4R-deficient mice leads to decreased T_H2-type cytokine production and prolonged T_H1 responses (Cheever et al., 1994; Kaplan et al., 1998; Jankovic et al., 1999). However, in certain murine strains, IL-4 deficiency was not enough to drive substantial increases in IFNγ production or to completely prevent development of a T_H2-type response during infection (Metwali et al., 1996; Rakasz et al., 1998). IL-10 and/or TGF-β were the dominant mediators T_H1 downregulation in IL-4 deficient mice (Rakasz et al., 1998). IL-4 deficient mice display reduced expression of IL-13, but the residual IL-13 may play a compensatory role for the loss of IL-4 expression (Chiaramonte et al., 1999b; McKenzie et al., 1999). IL-13 plays a similar role as IL-4 through binding to the IL-4 receptor and participates in granuloma formation, IgE induction, and inhibition of IFNγ production. In contrast to IL-4, IL-13 is also involved in the induction of collagen synthesis by granuloma fibroblasts through binding to the specific IL-13R (Chiaramonte et al., 1999a; Fallon et al., 2000a). T_H1 and T_H2 cytokines participate in cross-regulation of synthesis and function of the opposing cytokine response, therefore, it was initially unclear how the T_H2 response could be induced while the T_H1-type response that downregulates it was already actively mediating granuloma formation. One possibility is that IL-12 production from granuloma macrophages is decreased upon exposure to SEA (Boros, 1999; Todt et al., 2000).

Lacto-N-fucopentaose III (LNFPIII), a glycan component of SEA, has been shown to act directly on DC and monocytes to favor the development of the T_H2 response (Wang et al., 2010; Zhu et al., 2012). In comparison to LPS stimulation, LNFPIII induced an increase in the ratio of CD86/CD80 expression on DC, a similar pattern of chemokine expression, higher expression of IL-6 but no IL-12 from purified DC (Wang et al., 2010). Co-culture of these LNFPIII-stimulated DC with naive OT-II T_H cells led to dominant production of IL-4 and no IFNγ, but induced similar levels of IFNγ and cytolytic activity of OT-I $CD8^+$ T_C cells following co-culture in comparison with LPS-stimulated DC (Wang et al., 2010). LNFPIII was also shown to program macrophages to induce IFNγ production from NK cells by an IL-12-independent, but CD40/CD40L-dependent mechanism. Treatment with dextran conjugated to LNFPIII was successful at inhibiting experimental autoimmune encephalomyelitis (EAE) in mice through a mechanism involving alternative activation of $CD11b^+Ly\text{-}6C^{high}$ monocytes (Zhu et al., 2012). The LNFPIII-treated TCR transgenic 2D2 mice had elevated levels of IFNγ as well as IL-4, IL-5, IL-13, and IL-10 in response to immunization with MOG peptide in comparison with mice that received unconjugated protein (Zhu et al., 2012). These data demonstrate alternate pathways by which

LNFPIII elicits both IFNγ production from NK and CTL cells while also mediating a T_H2 and T_{REG} dominated responses from $CD4^+$ T cells, and suggest a mechanism for the induction of T_H2 cells in the face of a strong T_H1 immune response. The T2 ribonuclease, ω1, component of SEA was demonstrated to directly inhibit the activation of DC by LPS, suggesting that it is a critical factor in the inhibition of the T_H1 response (Everts et al., 2009; Steinfelder et al., 2009).

A study done using SEA stimulation of human basophils was one of the first in which IL-4 production by these cells was definitively shown (Falcone et al., 1996). Since that time it has become evident that basophils are not only capable of producing IL-4 but are also important antigen presenting cells that can initiate T_H2 responses (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). The IPSE/α-1 glycoprotein component of SEA was shown to activate IL-4 production from mouse basophils through a mechanism dependent on IgE antibodies and FcΣR expression (Schramm et al., 2007). Several other groups have also demonstrated IL-4 production in response to SEA by non-$CD4^+$ peritoneal exudate cells and activated granuloma eosinophils (Williams et al., 1993; Sabin and Pearce, 1995; Kullberg et al., 1996; Sabin et al., 1996b; Rumbley et al., 1999). IL-5 is a stimulatory cytokine for eosinophils and studies in IL-5 deficient mice have revealed a reduction in IL-4 production from ionomycin stimulated non-B, non-T cells from infected mice (Brunet et al., 1999). However, IL-4 production from SEA-stimulated or IgE crosslinked non-B, non-T cells of IL-5 deficient mice was comparable to normal mice and anti-IL-5 antibody treatment did not reduce granuloma sizes in infected mice (Sher et al., 1990). These non-T cell sources of IL-4 may be sufficient to supply the early IL-4 necessary to down regulate IL-12 and IFNγ production and to stimulate T_H2 cell differentiation.

THE ROLE OF T_H17 CELLS IN GRANULOMA PATHOLOGY

The first evidence of a role for IL-17 in schistosome granuloma formation came from a study in which blockade of the IL-12p40 receptor subunit shared by IL-12 and IL-23, but not the IL-12p35 receptor subunit that is specific to IL-12, led to decreased granuloma size (Rutitzky et al., 2005). Granuloma formation correlated with levels of IL-17 production in susceptible vs. resistant strains of mice and treatment of susceptible strains with anti-IL-17 neutralizing antibodies led to decreases in granuloma size (Rutitzky et al., 2005). It was later determined that SEA had direct stimulatory effects on IL-23 and IL-1 production by dendritic cells (Rutitzky et al., 2008; Shainheit et al., 2008). Mice with genetic deficiencies of the T_H1-specific transcription factor T-bet or IFNγ displayed enlarged schistosome granulomas and elevated IL-17 levels (Rutitzky et al., 2009; Rutitzky and Stadecker, 2011). The studies described above were mostly performed using immunization with SEA and complete Freund's adjuvant in the normally granuloma resistant C57BL/6 strain of mice. IL-4 and type 1 interferons, which may be suppressed by instillation of complete Freund's adjuvant, are normally elicited by schistosome eggs and are known inhibitors of T_H17 cells. It will be interesting to determine whether IL-4 and IFNα or IFNβ display inhibitory effects on IL-23 or IL-17 during schistosome infection in granuloma susceptible mice.

B LYMPHOCYTES AND ANTIBODIES

The dynamics of total B cell distribution and their roles during schistosome infection have also been intensely investigated. It was found that the absolute numbers of B cells in the spleen, lymph nodes and blood increased dramatically during the acute T_H2 phase of infection and remained high throughout the chronic infection (Chensue and Boros, 1979; El-Cheikh et al., 1998). The absolute number of T cells rose slightly in the same organs leading to a relative decrease in the number of T cells compared to B cells. This expansion of B cells was polyclonal in nature yielding both antigen-specific and non-specific antibodies, including autoreactive antibodies (Fischer et al., 1981; Lopes et al., 1990). SEA-specific immunoglobulin production begins following egg deposition and increases throughout the acute and chronic downmodulated phases of schistosome infection (Boros et al., 1975). Evidence of intra-granulomatous antibody production exists with IgG1 being the predominant antibody isotypes released from acute-phase granulomas and a mixture of IgG1, IgG2a, IgG2b, IgG3, and IgA released by chronic-phase granulomas (Boros et al., 1982). Intralesional production of IgM peaked at 12–16 weeks of infection while IgG production was highest at 20 weeks of infection (Boros et al., 1982). The role of antibodies in the pathogenesis of schistosome granuloma formation is complex, with several studies showing an active immune regulatory role of antibodies from infected individuals (Goes et al., 1991; Jankovic et al., 1997, 1998; Rezende et al., 1998). Immune complexes from the blood of chronically infected human patients were able to inhibit *in vitro* granuloma formation (Goes et al., 1991). This inhibition was dependent on the presence of the Fc portion of the immunoglobulin and could be reversed by treatment with indomethacin, indicating a role for prostaglandins in immune suppression mediated by immune complexes (Goes et al., 1991). Infection of mice deficient in either FcγR or FcΣR led to increased size and collagen content of acute-phase and chronic granulomas (Jankovic et al., 1997, 1998). SEA-specific antibodies may mediate granuloma downmodulation by arming FcR positive suppressor cells, or by neutralizing and sequestering antigens. Another mechanism by which immune complexes may mediate downmodulation is through inhibition of MHC Class II expression and disruption of antigen presentation (Rezende et al., 1998).

Several lines of evidence suggest that immune regulation and control of granuloma formation may be the primary function of B cells during schistosome infection. One study demonstrated that SEA-stimulated mesenteric lymph node cells of B cell-deficient (JHD) mice produced higher amounts of IFNγ and IL-12 cytokines and reduced amounts of IL-4 and IL-10 when stimulated with SEA (Hernandez et al., 1997). Irradiated splenocytes from JHD mice were more effective at eliciting a T_H1-type response from SEA-specific $CD4^+$ T cells. Another study in B cell-deficient (μMT) mice showed increased mRNA expression of T_H1 cytokines in B-deficient compared to wild-type mice (Ferru et al., 1998). These authors concluded that the B cell population was necessary to drive T_H2-type responses during infection. However, a different study using μMT mice demonstrated only a reduction in IL-4 production without differences in IL-5, IL-10, or IFNγ production (Jankovic et al., 1998). In contrast, infection

of BALB.Xid mice, which have a deficiency in B cell receptor signaling and severe reductions in the mucosal $CD5^+$ B cell compartment, revealed a defect in antigen-stimulated IL-10 production, reduced SEA-specific IgM and IgA titers, increased IFNγ and IL-4 production, elevated IgE and IgG1 titers, increased tissue egg burdens and higher mortality (Gaubert et al., 1999). Thus, B cells appear to play a central role in mediating the transition from T_H1 to T_H2 responses and in the regulation of the granulomatous response during acute and chronic infection. Antibody-independent regulatory mechanisms of B cells are discussed below.

SYSTEMIC IMMUNE REGULATION STIMULATED BY CHRONIC SCHISTOSOME INFECTION

As the T_H2 response progresses, SEA-induced and cytokine-mediated collagen synthesis by granuloma fibroblasts leads to increased hepatic fibrosis that continues through the remainder of infection (Boros and Lande, 1983). Schistosome granulomas reach peak size and cellularity at 8–10 weeks of natural murine infection. After 10 weeks, a spontaneous diminution of cytokine responses to SEA and decreased granuloma size (granuloma downmodulation) is observed leading to a less severe chronic stage of the murine disease by 14 weeks (Boros et al., 1975; Colley, 1975). Downmodulation is accompanied by cumulative hepatic fibrosis and elevated anti-SEA antibody titers (Boros et al., 1975). Splenectomy of infected mice at 8 weeks of infection led to increased granuloma size at 12 weeks of infection indicating that a splenic mechanism was involved in granuloma downmodulation (Hood and Boros, 1980). Subsequent studies were directed at identifying the splenic factor(s) involved in granuloma downmodulation.

The earliest findings showed that spleen cells from chronically infected mice were able to transfer downmodulation to acutely infected mice (Colley, 1976; Chensue and Boros, 1979). Depletion of T cells from spleen cell preparations demonstrated their role in transferring downmodulation (Chensue and Boros, 1979). A complex series of experiments followed in which the granuloma inducing properties of T cells were attributed to T_H cells, while granuloma and SEA-induced suppressive effects were attributed to both the T_H and $CD8^+$ cytotoxic T lymphocyte (CTL) subsets (Chensue et al., 1981; Weinstock and Boros, 1983; Ragheb and Boros, 1989; Fidel and Boros, 1990). However, a subsequent study of infection in CTL-deficient mice demonstrated normal granuloma formation during acute infection and normal downmodulation during chronic infection (Yap et al., 1997). These data suggested that although highly purified CTL were capable of regulating granuloma formation, their presence *in vivo* was not critical for controlling the immune response toward schistosome eggs. This indicated the importance of CTL-independent factors in mediating granuloma downmodulation that are now understood to involve the activation of regulatory populations of T and B lymphocytes as well as other mechanisms of immune suppression.

REGULATORY CYTOKINES: IL-10 AND TGF-β

The anti-inflammatory cytokine product of regulatory lymphocytes, IL-10, was recognized as an important mediator of the switch from T_H1- to T_H2-type response during schistosomiasis in the early 1990s (Sher et al., 1991; Oswald et al., 1992b). As is now a widely recognized property of IL-10, early neutralization studies led to increased MHC Class II and B7-1/B7-2 costimulatory molecule expression on granuloma macrophages and increased antigen presentation capacity for T_H1 cells (Flores Villanueva et al., 1993). In a subsequent study, recombinant IL-10 or IL-10/Fc fusion protein treatment of infected or egg-injected mice led to decreased granuloma formation, decreased IL-2 and IFN-γ production, and increased IL-4 and IL-10 production (Flores-Villanueva et al., 1996). In one study, mice treated with anti-IL-10 antibodies *in vivo* displayed increased hepatic and pulmonary granuloma size, increased eosinophilia, and elevated IFN-γ and IL-5 levels during acute infection (Boros and Whitfield, 1998). However, another study indicated that granuloma downmodulation was not impaired in IL-10 deficient mice (Wynn et al., 1998). The same group also showed that infections of IL-10/IL-4 and IL-10/IL-12 double deficient mice in comparison to mice with singular deficiencies led to severe immune polarizations to T_H1 and T_H2, respectively (Wynn et al., 1997). The double deficient mice died from very distinct pathologic mechanisms with the IL-10/IL-12 mice that were heavily skewed toward the T_H2 response having enlarged and highly fibrotic granulomas (Wynn et al., 1997; Hoffmann et al., 2000). These data indicated that IL-10 regulates the T_H2 as well as the T_H1 response toward schistosome antigens. Production of IL-10 in response to stimulation with SEA or its major glycan component, lacto-N-fucopentaose III (LNFPIII), has been attributed to T_H cells, B cells and monocytes (Sher et al., 1991; Velupillai et al., 1996a, 1997; Terrazas et al., 2001).

The role of TGFβ in granuloma pathology is even more complex than that of IL-10. TGFβ has an inhibitory effect on macrophages and T_H1 cells in the schistosome model (Oswald et al., 1992a; Qadir et al., 2001). Studies of inhibition of T_H cell responses and schistosome granuloma formation by TGFβ have the limitation that IL-10 and IL-4 were usually present (Oswald et al., 1992a; Qadir et al., 2001). In contrast, TGFβ in the context of proinflammatory cytokines acts as an important cofactor for T_H17 cell differentiation, and therefore, can contribute to the enhanced granuloma formation described in a previous section (Shainheit et al., 2008). TGFβ also induces the production of connective tissue components by fibroblasts and therefore may play a role in the development of fibrosis and portal hypertension seen in severely infected individuals (Wahl et al., 1997).

REGULATORY T LYMPHOCYTES

With the discovery that FoxP3 was the canonical transcription factor for regulatory T (T_{REG}) cells that produced IL-10 and TGFβ, it was of interest to study whether the previously observed effects of chronic granuloma-induced immune suppression were attributable to T_{REG} cells (Hori et al., 2003). In the natural infection model with *S. mansoni*, dramatic and progressive increases in the expression of FoxP3 mRNA were observed in the liver and spleen, with peak expression occurring at the 16 week granuloma downmodulated stage and highest in the liver (Singh et al., 2005; Taylor et al., 2006). Additional phenotypic markers of T_{REG} cells (CD103, GITR, OX40, and CTLA-4) were also elevated

at the mRNA level in the spleens of schistosome-infected mice (Layland et al., 2010). Over-expression of FoxP3 through retroviral gene transfer at the beginning of egg deposition resulted in increased expression of IL-4, IFNγ, and TGFβ but not IL-10, yet resulted in decreased granuloma formation (Singh et al., 2005). The expansion but low IL-10 expression of FoxP3$^+$ T_{REG} cells was also observed in a separate study of the natural infection model (Baumgart et al., 2006). Transfer of immune tolerance by T_{REG} derived from schistosome infected hosts was IL-10 independent (Pacifico et al., 2009). These data demonstrate that T_{REG} cells are activated and recruited by the schistosome granuloma, but that they are not necessarily the main source of IL-10 during infection, nor are they dependent on IL-10 for their immune suppressive functions.

REGULATORY B LYMPHOCYTES

The realization that B cells have regulatory capacity was initially demonstrated in response to sheep red blood cells and was associated first with regulatory antibody production and later linked to interactions with T cells (Stockinger et al., 1979; Zubler et al., 1980). Subsequent studies in mice and humans have demonstrated that B lymphocytes can regulate immune responses through antibody-dependent mechanisms as well as via direct cell–cell interactions and regulatory cytokine production (Klinker and Lundy, 2012). The schistosome granuloma model was one of the first natural infection models in which the regulatory properties of B lymphocytes were reported (Cheever et al., 1985). Mice that were genetically deficient in B cells failed to downmodulate granuloma formation around eggs deposited in the liver during chronic infection and had higher mortality rates than control mice with normal B cell development (Cheever et al., 1985). The regulatory properties of B cells in schistosome infection were originally attributed to the increased production of natural antibodies, which more recently have been demonstrated to play a role in the clearance of apoptotic cell debris and immune suppression (Chen et al., 2009; Vas et al., 2012). In addition, it was demonstrated that IL-10 production by B cells, particularly the CD5$^+$ B-1a cell subset, was induced *in vitro* by the schistosome glycan LNFPIII found in SEA (Velupillai and Harn, 1994; Velupillai et al., 1996a, 1997). A study of schistosome infection in Xid mice, which have a severe deficiency in CD5$^+$ B cells, demonstrated that this subset plays a role in regulating the production of IFNγ and IL-5 by T_H cells and in controlling granuloma size (Gaubert et al., 1999). IL-10 acts as an autocrine growth factor for CD5$^+$ B cells and both IL-12 and IFNγ inhibit the expansion of CD5$^+$ B cells during schistosome infection (Velupillai et al., 1996b). Redistribution of splenic CD5$^+$ B cells to Peyer's patches and mesenteric ganglia during schistosome infection has been reported, and may be one mechanism by which helminth infections regulate inflammatory bowel disease (El-Cheikh et al., 1998; Elliott et al., 2000).

Although most subsets of APC can produce IL-10 in response to stimulation through CD40 and toll-like receptors, two subsets of B cells have been identified as the major producers of IL-10 in mice, B10 and T2-MZP B cells (Evans et al., 2007; Yanaba et al., 2008). B10 cells have the surface phenotype CD5$^+$CD1d^{high} and may be the cells that responded to stimulation with schistosome LNFPIII in the previous study (Velupillai et al., 1997; Yanaba et al., 2008). Stimulation of IL-10 production by T2-MZP cells, which have the phenotype CD21^{high}CD23$^+$, has not yet been studied during schistosome infection. B10 and T2-MZP cells have been used to transfer immune suppression in several models of inflammation in mice, and work is being done to identify the equivalent of IL-10 producing B10 and T2-MZP cells in human peripheral blood (Blair et al., 2010; Iwata et al., 2011). Importantly, the regulatory responses induced by B10 and T2-MZP cells appear to be antigen specific, despite being linked primarily to IL-10 production, suggesting the requirement for antigen presentation in their regulatory function. A recent study demonstrated IL-10 producing CD1d^{high} B cells from the spleens of schistosome infected mice could transfer immune regulation in a mouse model of ovalbumin-induced asthma (Van Der Vlugt et al., 2012). A similar population of B cells was found to be prevalent in the peripheral blood of children infected with *S. haematobium* in Gabon (Van Der Vlugt et al., 2012). The effect of schistosome infection on responses to other infections and inflammatory stimuli will be discussed in greater detail in another section.

ACTIVATION-INDUCED CELL DEATH AND FasL$^+$ B LYMPHOCYTES

Interestingly, the CD5$^+$ B cells in schistosome-infected mice also expressed Fas ligand and were able to kill SEA-stimulated T_H cells by activation-induced cell death (AICD) (Lundy and Boros, 2002). AICD is a form of programmed cell death (apoptosis) that is an important mechanism of immune regulation during and after inflammatory reactions. Clusters of apoptotic cells were found in the spleens and granulomas of schistosome infected mice to a much greater extent than in spleens of uninfected mice (Estaquier et al., 1997). Culture of cells extracted from schistosome-infected mice demonstrated that CD4$^+$ and CD8$^+$ T cells spontaneously underwent apoptosis *in vitro* and were sensitive to apoptosis induction in response to IL-10 and mitogenic stimulation (Estaquier et al., 1997). A different study demonstrated increases in *in vivo* CD4$^+$ and CD8$^+$ T cell apoptosis from 4 to 7 weeks of infection compared to later time points, supporting the hypothesis that T_H1 cells were more susceptible to AICD than their T_H2 counterparts during schistosome infection (Fallon et al., 1998). In contrast, a study of human schistosomiasis indicated that T cells from patients with the less severe, intestinal form of disease were susceptible to apoptosis while T cells from patients with the more severe, hepatosplenic T_H1 form of disease were resistant to egg antigen-induced apoptosis (Carneiro-Santos et al., 2000). In a more prolonged model of natural murine *Schistosoma mansoni* infection, systemic sensitivity to T_H cell apoptosis commenced soon after egg deposition (5 weeks), and progressed throughout the early granulomatous stages (6–10 weeks), in parallel with increased inflammation, and persisted at lower levels throughout the chronic, downmodulated stage (12–16 weeks) of the infection (Lundy et al., 2001). Levels of apoptosis exceeded 20% of the total CD4$^+$ T cell population, which was far in excess of the expected number of antigen-specific cells, suggesting that a large number of bystander CD4$^+$ T cells may be eliminated during schistosome infection (Lundy et al., 2001). Culture of splenocytes from infected and uninfected mice with SEA led to T_H cell apoptosis only in cells isolated from

infected mice (Lundy et al., 2001). The three major lymphocyte populations of the spleen, $CD4^+$ and $CD8^+$ T cells as well as $CD19^+$ B cells expressed the death-inducing molecule Fas ligand (FasL, CD178) (Lundy et al., 2001). The staining of freshly isolated splenocytes from 5 to 16 weeks infected mice demonstrated that the number of surface $FasL^+$ cells correlated with the level of T_H cell apoptosis at each time point (Lundy et al., 2001).

FasL expression on $CD8^+$ and $CD4^+$ T cells is not surprising given that both T cell recognition of schistosome antigens and FasL up-regulation are mediated through the TCR. FasL expression by B cells was a much rarer finding, and the schistosome model provided some of the first evidence of the ability of isolated B lymphocytes to kill T_H cells in an SEA-dependent fashion (Lundy et al., 2001; Lundy and Boros, 2002; Lundy, 2009). Culture of splenocytes from 8 week-infected mice with SEA led to upregulated FasL expression on B and T cells and increased T_H cell apoptosis. Depletion studies indicated that the majority of T_H cell death was mediated by B cells rather than $CD8^+$ CTL (Lundy et al., 2001). In a follow up study, B cell FasL expression was observed primarily on the splenic $CD5^+$ B cell subset throughout schistosome infection and was even observed on splenic $CD5^+$ B cells in uninfected mice (Lundy and Boros, 2002). Purified $CD5^+$ B cells killed T_H cells from schistosome-infected mice at a low effector:target (0.5:1) ratio only when SEA was present (Lundy and Boros, 2002). FasL expression on $CD5^+$ B cells was stimulated by culture with SEA and further enhanced when IL-4 and/or IL-10 were added to the culture (Lundy and Boros, 2002). The induction of IL-10 production by $CD5^+$ B cells following exposure to schistosome glycans may act in an autocrine manner to induce higher FasL expression on these cells and suggests that $CD5^+$ B cells may have several mechanisms of immune suppression at their disposal (Velupillai et al., 1996a; Klinker and Lundy, 2012). Induced FasL expression and IL-10 production by $CD5^+$ B cells, which are well-documented to have autoreactive and polyreactive antibody specificities, may be an important mechanism behind the correlations between schistosome infection and protection from autoimmunity and asthma described below (Lundy et al., 2005; Lundy and Fox, 2009).

OTHER MECHANISMS OF IMMUNE REGULATION

Other cell types may also participate in downregulation of the immune response to schistosome eggs. Granuloma macrophages represent about 30 percent of the total cells in the lesion. Early in the response to infection, macrophages and monocytes are important in the elicitation of delayed-type hypersensitivity granuloma formation and have cytotoxic effects on the miracidiae (Boros and Warren, 1971a). However, as described above, exposure to components of SEA results in alternative activation of monocytes and macrophages as well as DC, and promotion of the transition to a T_H2 dominated immune response (Zhu et al., 2012). In addition, exposure of monocytes/macrophages to SEA leads to suppressive effects on SEA-induced T lymphocyte proliferation (Elliott and Boros, 1984). These macrophage suppressive effects may be attributable to SEA-induced production of IL-10, type 1 interferons and/or prostaglandin E_2 (Elliott et al., 1987, 1990; Atochina and Harn, 2005). An interesting study of the effects of schistosome infection on chemically-induced experimental colitis in mice demonstrated an immune regulatory effect of $F4/80^+$ macrophages that had migrated to the lamina propria (Smith et al., 2007). These macrophages were not induced by injection of schistosome eggs alone, and their mechanism of action was reported to be independent of alternative activation markers, interactions with T_H2 and T_{REG} cells, or production of TGFβ or IL-10 (Smith et al., 2007). Schistosome infection also induces production of IL-10 by DC that in turn can induce the activation of $FoxP3^+$ T_{REG} cells (Liu et al., 2011). These schistosome-induced DC transferred resistance to airway inflammation following adoptive transfer in an ovalbumin-induced model of asthma (Liu et al., 2011). Similar IL-10 production by DC and DC-dependent induction of T_{REG} cells was found following schistosome infection in the NOD mouse model of type 1 diabetes (Zaccone et al., 2009).

SCHISTOSOME INFECTION MODULATES IMMUNE RESPONSES TOWARD OTHER ANTIGENS

The systemic immune modulatory and regulatory pathways elicited by schistosome infection are not limited to antigens produced by adult schistosomes or their eggs (Harnett and Harnett, 2006; Helmby and Bickle, 2006; Kamal and El Sayed Khalifa, 2006; Zaccone et al., 2006). Bystander immune modulation by helminths has become a topic of great interest because of its negative impact on responses to vaccinations and pathogenic co-infections (Kamal and El Sayed Khalifa, 2006). With over 200 million estimated cases of schistosome infection worldwide, diminished responsiveness to protective vaccines and pathogenic infections poses a major public health hurdle, and justifies the support for global initiatives aimed at reducing schistosome infection (Borkow and Bentwich, 2000). Yet, as will be discussed, schistosomes and other helminths have been correlated to protection from allergies and autoimmunity by many epidemiological studies as well as experimental model systems and clinical trials (Kamal and El Sayed Khalifa, 2006; Zaccone et al., 2006; Maizels, 2009).

RESPONSES TO VACCINATIONS AND CONCURRENT INFECTIONS

The switch from T_H1- to a strong T_H2-dominated immune response that occurs during progression of schistosome infection and egg granuloma formation has a negative impact on the ability of the host to respond correctly to intracellular pathogens. As the infection becomes more chronic, the T_H2 response gives way to a highly regulatory immune response that can dampen both T_H1 and T_H2 immunity, leaving the host very susceptible to both intracellular and extracellular microbes. While this progression may be necessary to regulate liver toxicity and granuloma formation caused by the schistosome eggs (see above), it also causes significant morbidity and increased mortality in infected individuals. Schistosome infection has been shown to have preventive effects on immune responses elicited by vaccines against tetanus toxoid in humans and diphtheria toxin as well as Bacillus-Calmette-Guérin in mice (Sabin et al., 1996a; Haseeb and Craig, 1997; Elias et al., 2005). These examples illustrate altered humoral and cellular immune responses toward vaccines and will most likely translate to many other types of vaccinations including those against HIV, hepatitis viruses, *Mycobacteriae spp*.

and malaria, significant pathogens that coexist in schistosome endemic areas.

The interaction between schistosome and HIV infections is of particular importance as the overlapping endemic regions for these two infections persist. Cells from schistosome infected individuals are more susceptible to HIV-1 infection reportedly due to IL-4 or IL-10 induced expression of the HIV co-receptors CXCR4 and CCR5 on immune cells of schistosome infected subjects (Shapira-Nahor et al., 1998; Kalinkovich et al., 1999; Secor et al., 2003). Selective inhibition of the cytolytic response of $CD8^+$ T cells for the HIV Gag protein has also been noted in schistosome infected individuals (McElroy et al., 2005). These findings could be expected to increase cell–cell transmission and viremia in individual patients, and therefore, to increase the person to person transmission of HIV in schistosome endemic areas (Quinn et al., 2000). However, studies of HIV viral load and disease progression in schistosome infected individuals following de-worming have yielded conflicting results (Lawn et al., 2000; Wolday et al., 2002; Secor et al., 2004; Kallestrup et al., 2005). Susceptibility to HIV infection and response of the virus to de-worming is undoubtedly dependent on the stage and severity of the schistosome infection and the status of the immune response at the time of HIV exposure. In contrast, schistosome egg excretion is increased by T cell-mediated inflammation, and persons co-infected with HIV had marked decreases in the number of excreted eggs (Doenhoff, 1997).

Another major public health concern is co-infection of schistosome infected individuals with liver tropic viruses such hepatitis B and C (Kamal et al., 2004). Although there is evidence that schistosome infection does not increase the incidence of hepatitis infection in endemic areas for both diseases, co-infections in these regions are quite frequent (Larouze et al., 1987; Pereira et al., 1994; Angelico et al., 1997). Interestingly, a study of liver biopsies of patients infected with hepatitis C showed no significant impact of co-infection by schistosomes or the presence of egg granulomas on virus-induced pathology (Helal et al., 1998). However, a separate study focused instead on the liver pathology of schistosome infected individuals, showed that co-infection with hepatitis C led to much more severe liver damage including cirrhosis and cancers that were not found in the absence of HCV co-infection (Mohamed et al., 1998). Despite the lack of evidence that schistosome co-infection changes the course of liver pathology caused by hepatitis virus, some studies have shown differences in T_H cell cytokine responses toward viral antigens in schistosome co-infected people and viral persistence (Kamal et al., 2001; El-Kady et al., 2005). Schistosome co-infection suppressed the T_H1 response normally elicited by HCV infection (Kamal et al., 2001; El-Kady et al., 2005). An improvement was seen in the anti-viral response of a cohort of co-infected patients following treatment with praziquantel to remove schistosomes (Farghaly and Barakat, 1993). In an experimental model of hepatic virus infection, schistosome infected mice co-infected with LCMV had an influx of activated $CD8^+$ CTL cells expressing IFNγ into the liver, which led to down regulation of the granuloma T_H2 response (Edwards et al., 2005). The end result of LCMV co-infection was increased liver toxicity and viral replication in the schistosome infected mice (Edwards et al., 2005). Thus, schistosomes and hepatotropic viruses have a complex relationship that generally leads to alterations in immune responses to both pathogens, and worse prognosis.

Schistosome infections can also influence the immune response toward other pathogens. Mice co-infected with *Mycobacterium avium* and *Schistosoma mansoni* had a shift to T_H2 dominated cytokine responses toward mycobacterial antigens even when the *M. avium* infection was established first (Sacco et al., 2002). Similar results were seen in a co-infection model of *S. mansoni* and *M. leprae* in mice (Frantz et al., 2010). As noted above, schistosome infected mice responded poorly to vaccination with Bacillus-Calmette-Guerin, yet in the *M. leprae* study, vaccination with a DNA vaccine encoding the heat shock protein 65 molecule did induce a protective response in *S. mansoni* infected mice (Elias et al., 2005; Frantz et al., 2010). It remains to be seen whether such a strategy could be effective in co-infected humans, particularly those individuals who are also infected with the HIV virus. A study of the progression of active tuberculosis in HIV-infected individuals revealed a correlation between co-infection with schistosomes and increased active tuberculosis (Brown et al., 2006). A notable increase in susceptibility to malaria infection has also been described in people infected with schistosomes and other intestinal helminths (Nacher et al., 2002b; Sokhna et al., 2004). At the same time, schistosome infection may play a beneficial role in limiting the more severe cerebral form of malaria infection (Nacher et al., 2002a; Waknine-Grinberg et al., 2010). Co-infection of mice with *Plasmodium chabaudi* and *S. mansoni* led to reciprocal alterations in the immune response to either pathogen (Helmby et al., 1998). The widespread presence of schistosomes in areas that are endemic for these other major pathogens is likely to impede progress in designing effective vaccination and eradication strategies in the near future.

SCHISTOSOMES AND THE HYGIENE HYPOTHESIS

While major attempts at de-worming in schistosome endemic areas are important and worthwhile public health goals, there are substantial epidemiological and experimental data that indicate that this could lead to an increased risk for autoimmune diseases and allergy. The "hygiene hypothesis" asserts that the measurable increase in both T_H1 and T_H2 immune-mediated diseases that has occurred in the last century in developed nations is a result of improvements in sanitation that have led to increased sensitivity of the immune system to self-antigens and/or allergens (Gale, 2002; Airaghi and Tedeschi, 2004; Rook, 2012). Although the hygiene hypothesis was originally focused on the opposing roles of T_H1 and T_H2 immune responses that were established in childhood on later immune challenges, the hypothesis came under scrutiny when it was realized that both T_H1 (type 1 diabetes) and T_H2 (allergy) immune-mediated inflammatory diseases were rising in the same geographical areas and demographic groups (Stene and Nafstad, 2001). More telling was the fact that the prevalence of asthma and schistosome infections did not overlap despite both provoking heavily T_H2-biased immune responses (Yazdanbakhsh et al., 2001). Now that it is understood that chronic helminth infections also stimulate a highly regulatory immune response that affects both T_H1 and T_H2 reactions, a role for schistosomes and other worms in the hygiene hypothesis fits

better with the epidemiological data (Wynn et al., 1997; Araujo et al., 2004; Maizels, 2009). This intriguing correlation has led to series of experiments in mice as well as clinical trials to test the ability of helminths to modulate autoimmune diseases and asthma.

AUTOIMMUNE DISEASES AND SCHISTOSOMES

Type 1 diabetes (T1D) is an autoimmune disease that has risen in prevalence in developed and developing nations as hygiene has improved, and is rarely found in areas endemic for schistosomiasis (Zaccone et al., 2008). Cercarial infection of NOD mice that spontaneously develop T1D with *Schistosoma mansoni* prior to T1D disease development led to granuloma formation in the pancreas and a delay in hyperglycemia (Cooke et al., 1999). The same study showed that weekly injection of schistosome eggs led to suppression of anti-insulin IgG antibody production and decreased blood glucose levels in comparison to control NOD mice (Cooke et al., 1999). These effects were later shown to be dependent on the timing of administration and could be recapitulated by treatment with soluble antigens extracted from adult worms as well as with SEA (Zaccone et al., 2003). Splenic T cells from egg-sensitized NOD mice produced similar levels of IFNγ, but much higher amounts of IL-4, IL-5, IL-13, and IL-10 in response to SEA challenge than did cells from control mice (Zaccone et al., 2003). Unlike soluble antigens from adult worms, SEA cooperated with bacterial lipopolysaccharide (LPS) in mediating a shift away from IL-12 production and toward IL-10 production in bone marrow-derived DCs from NOD mice (Zaccone et al., 2003). Adoptive transfer of splenocytes from SEA-treated NOD mice failed to induce T1D in NOD. SCID recipients, an effect attributed to TGFβ-dependent induction of $FoxP3^+/GITR^+/CD25^+$ T_{REG} cells (Zaccone et al., 2003, 2009). Correlative studies involving schistosome infection have been reported in mouse models of Grave's disease and autoimmune arthritis (Nagayama et al., 2004; Osada et al., 2009; Song et al., 2011).

A series of experiments has been conducted in the EAE model of multiple sclerosis in which susceptible mouse strains are immunized with peptides derived from proteolipid protein or myelin oligodendrocyte glycoprotein emulsified in complete Freund's adjuvant. An initial study demonstrated that prior infection of mice with *Schistosoma mansoni* led to reduced incidence of EAE as measured by paralysis and delayed infiltration of $CD3^+$ T cells and $F4/80^+$ macrophages into the brain and spinal cord (La Flamme et al., 2003). These effects were attributed to reduced levels of TNFα, IFNγ and IL-12 produced by cells from schistosome-infected mice (La Flamme et al., 2003). Very similar results were obtained using schistosome eggs to pretreat mice prior to EAE disease induction (Sewell et al., 2003). In the latter study, egg injection within the first two days after induction of EAE led to a significant delay in the onset of paralysis but became less effective as the time after disease induction increased (Sewell et al., 2003). The EAE model can be mediated by either T_H1 or T_H17 cells, which are both sensitive to immune regulation by IL-4, and it was shown that the effects of schistosome eggs on EAE were dependent on the major signaling transcription factor downstream of IL-4 receptor, STAT6 (Sewell et al., 2003). Protection from EAE has also been demonstrated using either unfractionated SEA or purified LNFPIII derived from schistosome eggs (Zheng et al., 2008; Zhu et al., 2012). Despite the findings that a delay in exposure to schistosomes or their products can prevent therapeutic effects on EAE, the data in humans appears to be more promising. A small cohort of patients that had previously been diagnosed with MS and then developed eosinophilia related to intestinal helminth infection was identified in Argentina (Correale and Farez, 2007). These patients had significantly fewer exacerbations of MS upon 5-year follow up than matched control patients who were uninfected (Correale and Farez, 2007). The peripheral blood of the infected MS patients had a higher frequency of IL-10 and TGFβ producing cells than controls, and significantly reduced numbers of IL-12 and IFNγ producers (Correale and Farez, 2007). Further studies of this cohort indicated that IL-10 producing B cells were prevalent in the infected MS patients (Correale et al., 2008). B cells and DCs from infected patients expressed TLR2 at high levels and suppressed T_H1 and T_H17 cell cytokine production while promoting T_H2 cytokines after treatment with TLR2 ligands (Correale and Farez, 2009).

It has also been suggested that the greatly increased incidence of the inflammatory bowel diseases (IBD), ulcerative colitis and Crohn's disease, in developed countries may be due to reduced exposure to helminths including *Schistosoma mansoni* (Weinstock and Elliott, 2009; Elliott and Weinstock, 2012). The gut mucosa is a site of exposure of the host to many potentially harmful microbes, as well as a majority of non-harmful commensal organisms. In order to maintain a balanced immune response in the gut mucosa, there are specialized lymphoid structures and cell populations that generally have a highly regulatory phenotype (Rubtsov et al., 2008; Maynard and Weaver, 2009). The development of IBD is known to be strongly suppressed by IL-10 and regulatory T cells as evidenced by animal models involving circumvention of these mechanistic pathways (Rubtsov et al., 2008; Glocker et al., 2009). As noted above, both IL-10-producing $CD5^+$ B cells and $FoxP3^+$ T_{REG} cells are stimulated by infection with schistosomes. In particular, $CD5^+$ B cells are normally resident in the mucosa and have high regulatory potential due to inducible expression of IL-10, FasL, regulatory natural antibodies and other mechanisms of immune regulation (Klinker and Lundy, 2012). Direct experimental evidence of a role for schistosome and other helminth infections in the suppression of IBD has come from experimental mouse models (Smith et al., 2007; Weng et al., 2007). Mice that were infected with cercariae 8 weeks prior to the elicitation of colitis in the dextran sodium sulfate (DSS) model did not display weight loss, bloody stools or shortening of colon length as was detected in uninfected control mice (Smith et al., 2007). In addition to induction of IL-10 and TGFβ, neither of which was solely responsible for protection from colitis, schistosome infection led to increased infiltration of macrophages into the intestinal lamina propria that were required for immune suppression (Smith et al., 2007). Although exposure to schistosome eggs did not elicit protection from orally-induced DSS colitis, this treatment was effective in the trinitrobenzene sulfonic acid (TNBS) model that involves the more localized rectal administration of the eliciting agent (Elliott et al., 2003).

Similarly, administration of worm proteins extracted from adult schistosomes in the TNBS colitis model as much as 6 h after rectal administration of TNBS led to decreased inflammation (Ruyssers et al., 2009). The worm antigen treatment was accompanied by increased colonic expression of IL-10, TGFβ, and IL-5 but diminished expression of IFNγ, IL-12, IL-17, and IL-4 (Ruyssers et al., 2009). The epidemiological and experimental data outlined above was used as justification for Phase I open-label trials and Phase II randomized, controlled clinical trials using ingestion of ova from the pig whipworm, *Trichuris suis*, to treat ulcerative colitis (Summers et al., 2003, 2005). Oral intake of 2500 *T. suis* ova biweekly for 12 weeks led to clinical improvement in 43% of ova-treated patients compared to 17% improvement using the same measures in placebo-control recipients at the end of the 12 weeks treatment period (Summers et al., 2005). Therapy with *T. suis* eggs may have several advantages over similar treatment with eggs from other helminths since the *T. suis* eggs are easy to obtain and store, and do not permanently colonize humans or invade into tissues where they could become pathogenic (Weinstock et al., 2002).

ASTHMA

There was an early expectation that an association of increased incidence of allergic asthma and other atopic disease responses in individuals with schistosoma infections would exist, due to the strongly skewed T_H2 immune response environment induced by SEA. However, this was not the case and individuals with schistosoma infection were shown to have lower incidence of allergic asthma compared to those without (Yazdanbakhsh et al., 2001; Araujo and De Carvalho, 2006). While this effect does not appear to be limited to schistosomes, they are the most widely endemic infectious organism among helminths and the most thoroughly studied. Initial data suggested that the responses could be correlated to increased levels of IL-10 and subsequently to T_{REG} cells that are generated during chronic schistosoma infection (Cardoso et al., 2006; Yang et al., 2007). Interestingly, recent data immunizing animals to a specific schistosome antigen, Sm22.6, demonstrated that allergen specific IgE as well as T_H2 cytokines were reduced and correlated with generation of T_{REG} cells (Cardoso et al., 2010). However, subsequent additional studies have demonstrated that numerous B cell associated responses may also contribute to a regulatory environment for the development and regulation of allergic responses, including both B cell-derived IL-10 production and altered T cell activation (Amu et al., 2010; Van Der Vlugt et al., 2012). In these studies, B cell transfer experiments demonstrated that $CD1d^{hi}$ B cells from schistosome-infected animals could modulate allergen-induced responses in an IL-10-dependent manner that stimulated T_{REG} cells in the recipients (Amu et al., 2010; Van Der Vlugt et al., 2012). A similar population of $CD1d^{hi}$ IL-10 producing B cells was found in the peripheral blood of *Schistosoma haematobium*-infected Gabonese children compared to uninfected children (Van Der Vlugt et al., 2012). Importantly, in the latter study a reduction in the B_{REG} population was reduced after treatment of the schistosome infection (Van Der Vlugt et al., 2012). In some B cell transfer studies involving cells isolated from helminth infected mice, there were also notable suppressive effects on asthma that were independent of IL-10, but the mechanisms were not determined (Wilson et al., 2010; Van Der Vlugt et al., 2012). Using animal models of chronic allergic responses it was observed that lung $CD5^+$ B cells could express FasL and regulate allergic responses by a FasL-mediated mechanism similar to that previously observed in schistosome infection (Lundy and Boros, 2002; Lundy et al., 2005). What is not clear from these studies is how the regulated immune response controlled by B cells from schistosome infections is able to cross react to allergens and elicit non-specific regulatory functions. Interestingly, since B cells can provide a T_H2-skewed APC function during allergic responses, there may also be other aspects of B cell biology that alters the direction of T_H2 type allergic immune responses (Lindell et al., 2008). It is conceivable that one aspect of B cell regulation might be related to the ability to alter the cytokine environment and indirectly affect activation of regulatory T cells. Thus, the infection with Schistosoma has a systemic effect on the development of allergic responses that includes an interrelated generation of numerous regulatory lymphocyte populations, both B and T cells, that may impact several aspects of immunity including vaccination responses.

THERAPEUTIC POTENTIAL OF SCHISTOSOME-DERIVED IMMUNOMODULATORS

A challenge moving forward with efforts to eradicate schistosomes will be to find ways of selectively replacing their potentially beneficial immune modulatory effects on allergy and autoimmunity, while preserving immune responses toward other pathogenic microorganisms. The correlations between chronic exposure to schistosome eggs and immune downregulation have led to interest in finding egg-derived molecules that can therapeutically mediate suppression of immune responses (Harnett and Harnett, 2010). It has long been recognized that SEA is a major mediator of the shift in immune response from the proinflammatory and T_H1 reactions prevalent early after infection toward the T_H2-dominated response that prevails in the acute phase of egg granuloma formation. SEA is a highly complex mixture of components including proteins, nucleic acids, lipids, glycolipids, complex carbohydrates, and glycoprotein molecules that can have direct or indirect effects on both innate and adaptive immune responses. Schistosome LNFPIII and glycoprotein ω-1 have been particularly interesting candidate molecules that contribute to immune modulation, yet there may be many other molecules in SEA or derived from other helminths that could hold therapeutic potential (Harnett and Harnett, 2010).

LACTO-N-FUCOPENTAOSE III

The pentameric, sialyl $Lewis^x$ containing glycan, lacto-N-fucopentaose III (LNFPIII, CD15), is a major carbohydrate component of SEA (**Figure 2A**). It was originally identified by antibody binding on human tumor cells and later found to be expressed by many human adult and fetal tissues, particularly in the mucosa (Brockhaus et al., 1982; Combs et al., 1984; Velupillai and Harn, 1994). As described above and shown in **Figure 2B**, the LNFPIII found in SEA was originally shown to have direct effects on the stimulation of IL-10 production by $CD5^+$ B cells *in vitro* (Velupillai et al., 1997). LNFPIII also drives a strong T_H2 response toward protein antigens with which it is conjugated when injected

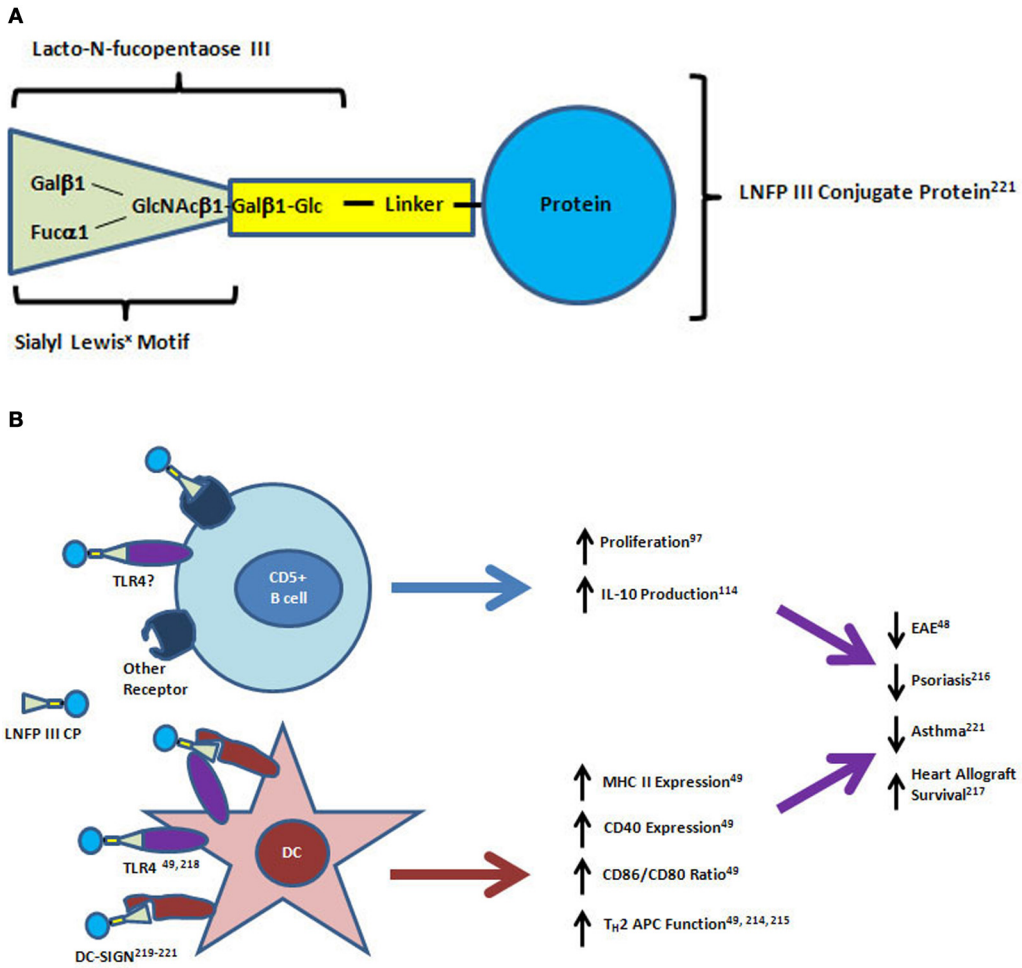

FIGURE 2 | Structure and mechanisms of action of lacto-N-fucopentaose III protein conjugates on CD5+ B cells and dendritic cells. (A) The lacto-N-fucopentaose III (LNFPIII) molecule is a pentameric glycan consisting of the trimeric sialyl Lewis^x motif (green triangle) linked to a beta-galactose and glucose dimer. For *in vitro* and *in vivo* studies, the glycan conjugated proteins have been purified from soluble egg antigen (SEA) or manufactured by linkage to a carrier protein molecule (bovine serum albumin, dextran, ovalbumin) using biochemical crosslinkers. **(B)** LNFPIII conjugates have direct effects on proliferation and IL-10 production by CD5$^+$ B cells. Dendritic cells (DC) respond to LNFPIII with altered antigen presenting capacity that favors T_H2 induction. Activation of DC by LNFPIII is mediated through TLR4 and/or DC-SIGN, while the receptors for LNFPIII on CD5$^+$ B cells have not yet been identified. Treatment of mice with LNFPIII protein conjugates has led to decreased disease progression in experimental autoimmune encephalomyelitis (EAE), asthma and psoriasis models, and led to prolonged heart allograft survival in a transplantation model. DC-SIGN, dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin; TLR4, toll-like receptor 4; APC, antigen presenting cell.

into mice (Okano et al., 2001; Faveeuw et al., 2002). Subcutaneous injection of a dextran-conjugated LNFPIII compound into psoriasis susceptible fsn/fsn mice prior to disease onset was shown to have a preventive effect on the formation of skin lesions (Atochina and Harn, 2006). LNFPIII has also been shown to be therapeutic in a mouse model of multiple sclerosis, and it prolonged the survival of allogeneic heart transplants in mice (Dutta et al., 2010; Zhu et al., 2012). LNFPIII skews the antigen presenting capacity of dendritic cells (DC) toward CD4$^+$ but not CD8$^+$ T cells (Wang et al., 2010). Treatment of spleen-derived DC from naïve mice with LNFPIII led to increases in MHC Class II and CD40 surface expression similar to the effects seen after bacterial lipopolysaccharide (LPS) treatment (Wang et al., 2010). However, LNFPIII induced higher expression of CD86 and lower expression of CD80 than LPS resulting in a dramatic difference in the ratio of these agonists for CD28 and CTLA4 molecules expressed by T cells (Wang et al., 2010). In coculture experiments, the LNFPIII treated DC preferentially induced IL-4 production from OT-II transgenic T_H cells isolated from the spleens of naïve mice (Wang et al., 2010). Details of the signaling effects downstream of LNFPIII treatment in DC has recently been well reviewed (Harnett and Harnett, 2010). LNFPIII signaling is dependent on TLR4, the receptor for LPS, but downstream signaling is significantly different between the two ligands particularly in the ERK and MAP kinase pathways and the mechanism of activation of the transcription factor NFκB (Thomas et al., 2005; Wang et al., 2010). In contrast to LPS stimulation, LNFPIII signals independently of MyD88 and does not induce degradation of the inhibitor of

NFκB (Thomas et al., 2005). A putative mechanism for this difference involves the binding of LNFPIII to C-type lectin receptors such as DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) by the Lewis^x motif (Van Liempt et al., 2004, 2007; Singh et al., 2009). It has been shown that activation of C-type lectins through binding of antibodies that are glycosylated with Lewis^x containing glycans is a mechanism of immune suppression induced by IVIG treatment, a therapy for immune thrombocytopenic purpura and other diseases (Anthony et al., 2008, 2012).

GLYCOPROTEIN ω-1

In the case of treatment for autoimmune diseases that are mediated by T_H1 or T_H17 immune responses, it may be sufficient to skew the response toward the T_H2 cytokine, IL-4, which has an inhibitory effect on IL-12 and IL-17 production. The SEA-derived T2 ribonuclease omega-1 (ω-1) has a direct effect on antigen presenting cells that supports T_H2 polarization (Everts et al., 2009, 2012; Steinfelder et al., 2009). Purified and recombinant ω-1 suppressed the ability of LPS to induce IL-12 secretion from DC as well as their ability to induce IFNγ-producing T_H1 cells in coculture experiments (Everts et al., 2009; Steinfelder et al., 2009). Injection of purified ω-1 into 4get/KN2 mice demonstrated that ω-1 could prime T_H2 responses *in vivo* and further experiments showed that T_H2 induction was independent of the IL-4R suggesting that the effects of ω-1 on T_H2 priming were direct (Everts et al., 2009). Like LNFPIII, ω-1 is glycosylated with sialyl Lewis^x motifs, and the T_H2 inducing capacity of ω-1 was shown to be dependent on glycosylation as well as the ribonuclease activity of the protein (Meevissen et al., 2010; Everts et al., 2012). Treatment of DC with SEA or purified ω-1 glycoprotein caused changes to the adherence of DC to plastic dishes and disrupted interactions with antigen-specific T_H cells (Steinfelder et al., 2009). Antibody neutralization and studies performed in mice with deficiencies in the mannose receptor indicated that ω-1 was dependent on the mannose receptor, but not on DC-SIGN, for uptake into DC *in vitro* and T_H2 polarization *in vivo* (Everts et al., 2012). Treatment of NOD mice with ω-1 led to global increases in the proportion of T_H1, T_H17, and T_H2 cells compared to untreated control mice, as well as a marked induction of FoxP3$^+$CTLA4$^+$ T_{REG} cells (Zaccone et al., 2011). The induction of FoxP3 expression by ω-1 was dependent on the functions of TGFβ and retinoic acid (Zaccone et al., 2011). It has not been reported whether treatment with ω-1 causes reduced or delayed development of diabetes in the NOD mice. Thus, both the glycoprotein ω-1 and the glycan LNFPIII components purified from SEA have demonstrable effects on adaptive immune parameters making each molecule a strong candidate for development of helminth-based immune therapeutics.

SUMMARY AND CONCLUDING REMARKS

Although nearly every person may have been expected to be infected by a helminth in their lifetime just a century ago, heroic eradication efforts have all but eliminated helminth infection from many areas and brought the infection rate down to an estimated 10–20% of people worldwide. This has led to vast improvements in the quality of life for billions of people, and will have great benefits for combatting other major infections. Several regulatory populations of lymphocytes are activated as the infection becomes chronic and these diminish the responses of schistosome antigen-specific effector T helper cells, and limit granuloma formation around newly deposited eggs. The chronic nature of schistosome-induced immune modulation can also affect immune responses toward other antigen sources, leading to poorer reactions to subsequent infections by other microbes. Also accompanying the shift in worm infection rates have been measurable increases in the rates of hyper-inflammatory diseases including allergies, asthma, and many autoimmune diseases. Chronic helminth infections may drive immune suppression toward allergens and self-antigens, thus potentially explaining the lower incidence of allergies, asthma and autoimmunity in schistosome endemic areas. Studies of the cellular and molecular mechanisms underlying schistosome-induced immune suppression in mice have supported the importance of regulatory T lymphocytes and the effector cytokine TGFβ as mediators of suppression. The schistosome infection model has also been instrumental in the discovery of immune regulatory functions of B cells including IL-10 production, and expression of the death-inducing molecule Fas ligand. IL-10-producing B cells may mediate cooperative effects with regulatory T cells, while FasL$^+$ B cells have been shown to have antigen-specific killer effects on T helper cells in models of asthma and autoimmunity. The data from adoptive cell transfer studies gives striking evidence that schistosomes do have cross-regulatory effects on autoimmunity and asthma. The goal as we move forward will be to find ways to mimic or replace the protective aspects of helminth infections, while continuing to improve hygienic conditions in endemic areas.

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The *Schistosoma* granuloma: friend or foe?

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Infection of man with *Schistosoma* species of trematode parasite causes marked chronic morbidity. Individuals that become infected with Schistosomes may develop a spectrum of pathology ranging from mild cercarial dermatitis to severe tissue inflammation, in particular within the liver and intestines, which can lead to life threatening hepatosplenomegaly. It is well established that the etiopathology during schistosomiasis is primarily due to an excessive or unregulated inflammatory response to the parasite, in particular to eggs that become trapped in various tissue. The eggs forms the *foci* of a classical type 2 granulomatous inflammation, characterized by an eosinophil-rich, $CD4^+$ T helper (Th) 2 cell dominated infiltrate with additional infiltration of alternatively activated macrophages (M2). Indeed the sequela of the type 2 perioval granuloma is marked fibroblast infiltration and development of fibrosis. Paradoxically, while the granuloma is the cause of pathology it also can afford some protection, whereby the granuloma minimizes collateral tissue damage in the liver and intestines. Furthermore, the parasite is exquisitely reliant on the host to mount a granulomatous reaction to the eggs as this inflammatory response facilitates the successful excretion of the eggs from the host. In this focused review we will address the conundrum of the *S. mansoni* granuloma acting as both friend and foe in inflammation during infection.

Keywords: *Schistosoma mansoni*, granuloma, inflammation, fibrosis, immunology

Schistosomiasis is a major chronic disease of humans in endemic regions. The schistosome species of major medical relevance to man are *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*. In this review we will focus on granulomatous inflammation following *S. mansoni* infection. Whilst in the majority of cases people infected with *S. mansoni* are relatively asymptomatic, or show restricted morbidity associated with intestinal inflammation and fibrosis, a minority of infected individuals develop a severe hepatosplenic schistosomiasis (HS). HS is characterized by hepatic fibrosis, hepatosplenomegaly, and portal hypertension, and can result in death in the absence of medical attention. A central feature of the pathology associated with *S. mansoni* infection is the development of granulomatous inflammation around parasite eggs that become trapped in tissue, in particular the liver and intestines. The host's immune response generated against parasite antigens plays a critical role in both dictating the severity of tissue inflammation and associated disease. Paradoxically, the host immune response to the parasite also facilitates parasite replication and survival. This review will focus on the immunobiology of the egg-associated granuloma elicited during *S. mansoni* infection and will address the conundrum of the *Schistosoma* granuloma eliciting inflammation that acts as both friend and foe during infection.

THE IMMUNOPATHOGENESIS OF *SCHISTOSOMA MANSONI*

Humans become infected with *S. mansoni* following exposure to water contaminated with skin penetrating cercariae. The *S. mansoni* cercariae are highly motile organisms able to enter the host *via* the penetration of intact skin. The cercariae transform into schistosomula after entering the skin and migrate *via* the vasculature and lymphatics through the lungs to the hepatic portal system. Schistosomula differentiate to male and female schistosomes, pair, and migrate to the mesenteric venous plexus, where adult worms can live for 5–10 years. Female schistosomes produce ∼300 eggs each day that are laid in mesenteric circulation. The eggs are viable, metabolically active organisms, and are highly antigenic. *S. mansoni* eggs adhere to the endothelium of mesenteric blood vessels and evoke inflammation leading to a granulomatous response that is necessary for translocation into the intestinal lumen and excretion in the feces (DeFranco et al., 2007). Indeed eggs have been shown to preferentially enter the Peyer's patches within the intestinal wall to facilitate egress of eggs to the intestinal lumen (Turner et al., 2013). Eggs that pass through the intestinal wall are excreted in the feces and if deposited in fresh water, may infect an appropriate species of snail, thus propagating the life cycle. However, some of the eggs may also become lodged in the host's intestine, liver, or other sites, where they can cause the morbidity and mortality associated with schistosomiasis *mansoni*.

Clinical signs of schistosomiasis are dependent on the maturation stage of parasites and their eggs. In humans, acute infection is characterized by a debilitating febrile illness (Katayama fever) that usually occurs before the appearance of eggs in the stool, having a peak 6–8 weeks after infection. In chronic disease, eggs trapped in various tissues evoke the formation of granulomatous inflammation, which along with the ensuing fibrosis cause the majority of pathological conditions. In individuals that develop HS, liver portal tract fibrosis leads to obstructive vascular lesions, portal hypertension, ascites, and fatal bleeding from esophagogastric

varices. Collectively, granulomatous inflammation around parasite eggs is a cardinal feature of schistosomiasis *mansoni* and the egg-associated pathology is central to the morbidity and indeed mortality that occurs in infected humans.

The use of animal models has facilitated the advancement of our understanding of the immunopathology during *S. mansoni* infection, with the mouse the most widely used species. It must be stressed that *S. mansoni* infection of mice does not faithfully recapitulate all aspects of human schistosomiasis (Fallon, 2000). Crucially, mice do not appear to develop portal tract fibrosis (Symmers' pipe stem fibrosis) that is associated with morbidity in humans, instead pathology in mice is primarily associated with a granulomatous responses to parasite eggs trapped in the host tissue, primarily in the liver and intestines. Recently, an interesting caveat to the use of inbred laboratory mouse strains was shown, with *S. mansoni* infection of wild outbred mice leading to more marked disease than inbred strains. The observed increased pathology in outbred animals was specifically associated with interleukin (IL)-1 elicited IL-17 producing $CD4^+$ T helper (Th)17 cells (Smith et al., 2011). Nevertheless mouse studies, in particular, the recent use of gene knockout or transgenic animals, has made fundamental advances in understanding the mechanisms of immunopathology of schistosomiasis.

Following infection of mice in the first 3–5 weeks, during which the host is exposed to migrating immature parasites, there is immune activation with a marked type 1 immune response, with increased Th1 cells and release of IL-12 and interferon (IFN)-γ. While the immune response during the initial weeks of infection with *S. mansoni* is strongly type 1-mediated, and primarily targeted against the worm antigens, it should be noted that type 2 responses are also primed. As the parasites mature, mate, and begin to produce eggs after 5–6 weeks, the immune response alters markedly alters, leading to a decrease in the type 1 immune component and concomitant emergence of a potent type 2 response (Pearce and MacDonald, 2002). The switch to a type 2-mediated response from 5 to 6 weeks post infection is a consequence of egg-production by mature female worms. *S. mansoni* eggs are potent inducers of type 2 responses when injected into naive mice (Vella et al., 1992). Furthermore, soluble egg-antigens (SEA) or antigenic egg secretions also induced a marked type 2 response. The egg-antigen stimulated type 2 response leads to Th2 cell expansion, production of IL-4, IL-5, and IL-13 accompanied by an upregulation in immunoglobulin (Ig) E levels and circulating eosinophils. The peak of this type 2 response corresponds with the maximal cell response against the egg and is closely associated with the magnitude of granulomatous inflammation surrounding the egg. During the chronic phase of infection, after ∼3 months, there is a marked decrease in the magnitude of the Th2 response and a state of hyporesponsiveness emerges. The potential for the Th2 response to lead to controlled chronic disease is part of a dynamic association between Th1, Th17, and T regulatory cells regulating disease severity during *S. mansoni* infection. For example, intestinal-associated $CD4^+CD25^+FoxP3^+T_{regs}$, which expand during chronic, schistosome-induced colitic inflammation, are capable of modulating Th2 *via* IL-4 suppression (Turner et al., 2011). More relevantly, a similar Th/reg cytokine interplay may also occur in man (Mbow et al., 2013).

IMMUNE DEPENDENCE OF GRANULOMA FORMATION

The classic phenomenon associated with *S. mansoni* infection is the formation of multi-cellular granulomatous inflammation surrounding eggs trapped in various tissues (**Figure 1**). The granulomatous response to the egg is primarily orchestrated by $CD4^+$ T cells. However, $CD8^+$ T cells, B cells, and M2 macrophages have also been shown to play a role in regulating granuloma formation (Fallon et al., 1998; Jankovic et al., 1998; Herbert et al., 2004). In addition, eosinophils also form a prominent constituent of the granuloma (Moore et al., 1977). However, while marked eosinophil infiltration is a cardinal feature of the *Schistosoma* granuloma (Lenzi et al., 1987), the actual function of eosinophils in the granuloma is not known. The generation of tissue eosinophilia during *Schistosoma* infection of mice is mediated by type 2 cytokines, such as IL-5 and IL-13, (Sher et al., 1990; Chiaramonte et al., 1999; Fallon et al., 2000; Reiman et al., 2006). However, using two transgenic mouse strains deficient in eosinophils, there was no marked defect in worm burden, granuloma formation, and liver fibrosis following *S. mansoni* infection (Swartz et al., 2006). Further work is required to fully elucidate

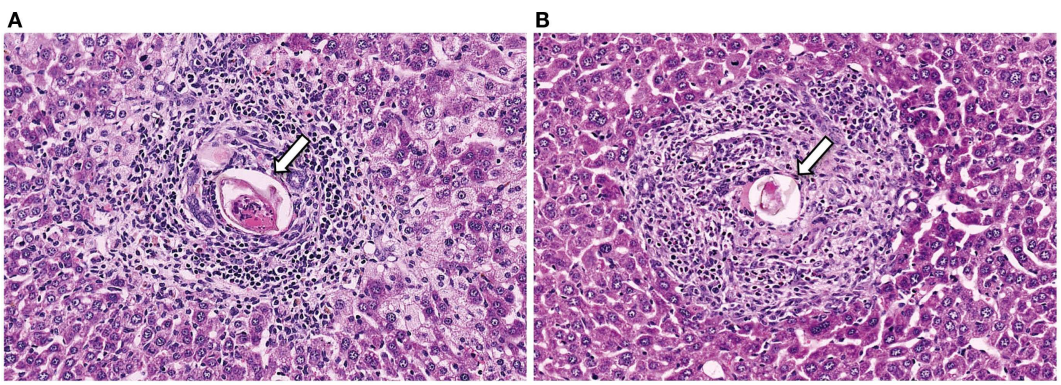

FIGURE 1 | Representative histology sections (stained with H&E) of livers from a $CD4^+$ T cell depleted (A) and normal (B) mouse, with arrows to indicate the *S. mansoni* egg.

the functions of eosinophils within the *Schistosoma* granuloma. Similarly, while mast cells are also present within the *Schistosoma* granuloma (Weinstock and Boros, 1983; Lenzi et al., 1987), the actual involvement of such cells in the formation of the granuloma around the egg and subsequent resolution is not known.

The importance of T cells in the generation of the granuloma was initially shown in nude mice and animals subjected to T cell depletion; with T cell deficient mice having impaired granuloma formation around eggs (Byram and von Lichtenberg, 1977; Doenhoff et al., 1981). A dominant role for $CD4^+$ T cells in granuloma formation was shown using depleting monoclonal antibodies (Mathew and Boros, 1986; Fallon et al., 1998). While it could be anticipated that the attenuated granuloma surrounding the egg in $CD4^+$ T cell-deficient mice would lead to less pathology, the opposite occurred. Indeed there is striking mortality in *S. mansoni*-infected mice with a compromised immune system (**Table 1**). This highlights the paradox: the granuloma that forms to encapsulates the egg can lead to pathology but the granuloma also functions to protect the host.

S. mansoni infection of mice with $CD4^+$ T cells depleted develop an acute fatal disease, with animals dying from weeks 4–6 after infection; coincident with egg deposition in tissue (Fallon et al., 1998). In immunologically intact mice, the eggs that are deposited in the liver are encapsulated within the granuloma with hepatocytes outside the granuloma are overtly normal (**Figure 1**), with such mice having normal liver function. In contrast, in the absence of $CD4^+$ T cells there is a limited granulomatous response, with the cellular infiltrate around the egg being neutrophil dominated as opposed to the eosinophil-rich granuloma observed in immunologically intact mice (**Figure 2**). Furthermore, without an intact functional granuloma there is extensive microvesicular damage to hepatocytes, and a consequential elevation in serum transaminase levels consistent with hepatocyte damage. Grossly, the hepatic steatosis is evident with the fat-laden white appearance of the liver in immune suppressed infected mice (**Figure 3**).

Therefore formation of a granuloma around the egg can be perceived as functioning to sequester egg secretions that can cause hepatotoxicity (Dunne et al., 1991). In addition to liver specific pathology, the absence of a functional granuloma in immune suppressed mice also leads to an inability to efficiently excrete eggs in feces, and consequentially eggs are trapped in the intestines leading to inflammation. Indeed this phenomenon of immune dependence of egg excretion (Doenhoff et al., 1981), illustrates the novel usurping of immunity by the parasite whereby the egg granuloma functions to induce a specific host immune response resulting in the translocation of the egg through the intestinal wall to be excreted in the feces. It is noteworthy that in other chronic granulomatous diseases, specifically tuberculosis, it is suggested that instead of limiting bacterial proliferation, the granuloma may actually benefit the bacteria (Ramakrishnan, 2012).

Crucially, these experimental observations on a role for $CD4^+$ T cells in facilitating egg excretion in *S. mansoni*-infected mice also occur in humans. Karanja et al. examined egg excretion, i.e., detection of eggs in the feces, in a cohort of *S. mansoni*-infected individuals in Kenya that were seronegative or seropositive for human immunodeficiency virus (HIV). There was a positive association between egg excretion rates and levels of circulating CD4 in HIV^+ patients, inferring a functional immune response was needed for egg excretion in man (Karanja et al., 1997). This may be *S. mansoni* specific phenomenon, or related to infection intensity, as in another study in Zimbabwe there was no such association between immune status and egg excretion in patients that were predominately infected with *S. haematobium* (Kallestrup et al., 2005).

The earlier studies in mouse models established an essential role for $CD4^+$ T cells in granuloma formation. More recently, the role of Th1 cellular cytokines [such as Tumor Necrosis Factor (TNF)-α, IFN-γ, and IL-2], Th2 (such as IL-4, IL-5, IL-9, and IL-13), Th17 (IL-17), and T regulatory cells in granuloma formation have been elucidated (Singh et al., 2005; Rutitzky and Stadecker, 2011). For example, mice deficient in type 1 cytokines such as IFNγ and IL-12p40 show little alteration in pathology, whilst mice deficient in certain type 2 cytokines are unable to generate a granuloma and may develop exacerbated pathology (Wynn and Cheever, 1995). Thus IL-4 deficient mice have impaired granuloma formation and develop acute fatal cachexia (Brunet et al., 1997). Indeed in mice that are tolerized to egg-antigens, a type 1 biased response is evoked leading to hepatotoxicity and death (Fallon and Dunne, 1999). Furthermore, in the absence of IL-4 alone, or both IL-4 and IL-13, there is acute mortality with impaired

Table 1 | Summary of the consequence of *S. mansoni* egg infection in immunologically intact and immune compromised (e.g., $CD4^+$ T cell depleted) mice.

	Liver				Intestine			Systemic			
	Gr[a]	Eo[b]	Fibrosis	Hepatocyte damage	Egg excretion	Eo[b]	Pathology	Antibody responses	T cells	Endotoxemia	Mortalities[c] (%)
Immunologically intact mice	+	+	+	−	+	+	−	+	Th2/Treg	−	<5
Immune compromised mice	±	−	±	+	−	−	+	±	Th1/17	+	100

[a] *Gr, granulomatous inflammation.*

[b] *Eo, tissue eosinophilia.*

[c] *Mortalities are expressed as percentage (ranges) of mice dead by 56 days after an acute infection.*

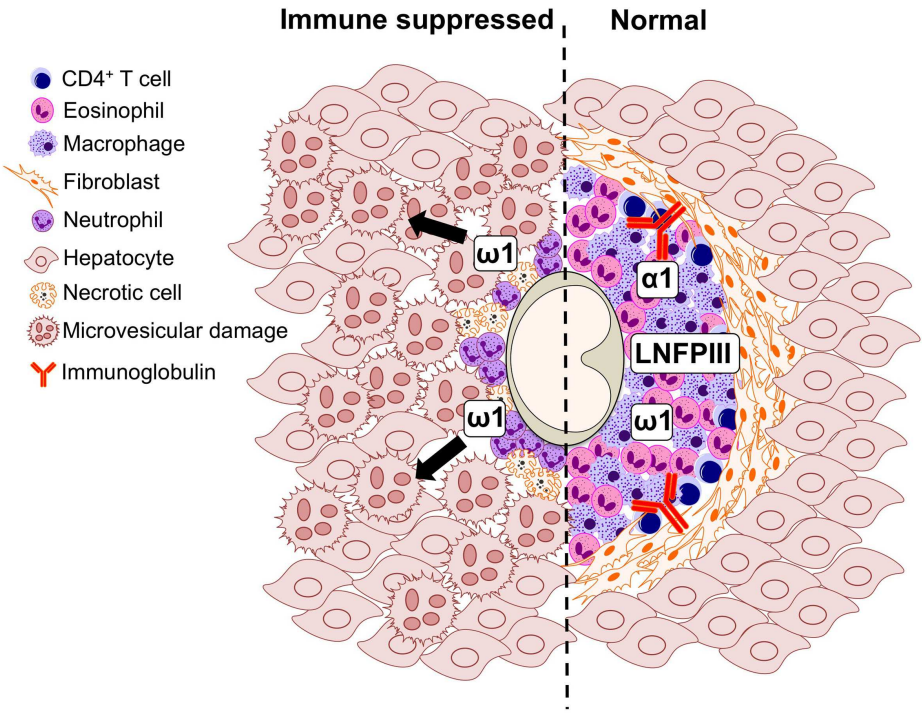

FIGURE 2 | Graphical representation of the cell populations involved in the formation of the *S. mansoni* egg-induced liver granuloma from an immune suppressed (*left*) and immunologically intact mouse (*right*).

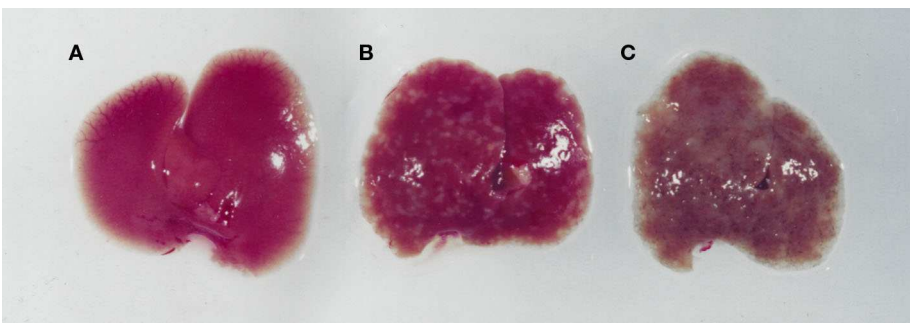

FIGURE 3 | Images of livers from an uninfected (A), an *S. mansoni*-infected immune competent (B), and immune suppressed (C) mouse. While an infected immunologically intact mice has granulomas in the liver **(B)**, in an immunodeficient mouse **(C)** there is marked whitening of the liver due to microvesicular hepatosteatosis.

egg excretion leading to endotoxemia (Fallon et al., 2000). Thus the generation of a functional granuloma is essential for a balanced cellular immune response ensuring survival of mice during infection with *S. mansoni*.

While a major focus has been on facets of adaptive immunity contributing to the formation of the egg granuloma, in recent years there is an increasing recognition that innate immunity also contributes. The generation of type 2 immunity by schistosome eggs requires antigen-presenting cells, such as dendritic cells (DC), processing and presenting schistosome egg-antigens (MacDonald et al., 2002). Indeed, depleting $CD11c^+$ DCs during active *S. mansoni* infection severely impairs Th2 responses, suggesting that DCs are critical for Th2 induction (Phythian-Adams et al., 2010). DCs are equipped with an array of pattern recognition receptors (PRRs), including C-type lectin receptors (CLRs) and Toll-like receptors (TLRs), in order to recognize and differentiate between pathogens by binding pathogen-associated molecular patterns (PAMPs) and instruct the immune system to mount a dedicated response. In order to induce a Th2 response, SEA interferes with TLR-mediated DC activation (Kane et al., 2004). SEA can suppress maturation and cytokine production of human and murine DCs induced by activation with the TLR4 ligand LPS and

the TLR3 ligand poly-I:C (MacDonald et al., 2002; Kane et al., 2004; van Liempt et al., 2007).

More recently, it has been shown that egg-antigens can also activate the NLRP3 inflammasome, in combination with a TLR agonist, leading to a release of IL-1β (Ritter et al., 2010). Such inflammasome activation modulates the immune response during *Schistosoma* infection, with mice deficient in NLRP3 developing smaller and more fibrotic granulomas (Ritter et al., 2010). In active infection it is feasible to conclude that TLR agonists such as LPS may leak through the intestinal wall and associate with egg-antigens to co-activate the inflammasome, leading to the release of IL-1β, which is essential for schistosome-related immunomodulation (Guo et al., 2009; Ritter et al., 2010). In this context it is relevant that in mice strains that develop more severe disease during *S. mansoni* infection, IL-1 receptor-associated kinase-like 2 (IRAK-2) was identified as a novel regulator of IL-1-induced pathogenic Th17 cells in schistosomiasis (Smith et al., 2011). The emergence of the importance of the inflammasome in the generation of granulomatous inflammation highlights the vital role for the innate immune response to the egg in generating the granuloma.

THE EGG GRANULOMA AND GENESIS OF FIBROSIS

A characteristic of *S. mansoni* infection is the development of fibrosis within the portal tracts of man and in mice within the egg granuloma. This pro-fibrotic property of the *S. mansoni* granuloma was used to identify that IL-13 was the dominant Th2 cytokine responsible for the development of liver fibrosis. While hepatic fibrosis is impaired in *S. mansoni*-infected mice unable to signal through IL-4Rα (*Il-4rα*$^{-/-}$), it is ablated in mice treated with soluble IL-13Rα2-Fc and also fails to develop in mice deficient in IL-13 (*Il-13*$^{-/-}$) (Chiaramonte et al., 1999; Jankovic et al., 1999; Fallon et al., 2000). In addition, *in vitro* studies have demonstrated the ability of IL-13 to directly stimulate collagen production in fibroblasts (Chiaramonte et al., 1999). The fibrogenic role of IL-13 involves the cytokine, together with IL-4, inducing the expression of arginase in macrophages *via* M2 polarization. Arginase uses L-arginine as a substrate to make L-ornithine, which is converted by ornithine aminotransferase to proline, a crucial amino acid for the production of collagen and the development of fibrosis (Hesse et al., 2001). The major function of Arg-1 is to down-modulate granulomatous inflammation in the liver and intestine and to slow the progression of Th2-dependent fibrosis in chronically infected mice (Pesce et al., 2009). The high expression of Arg-1, Ym-1, and FIZZ1 in granulomatous tissue reflects the large population of M2 macrophages and fibroblasts in the granuloma (Hesse et al., 2001). The presence of M2 macrophages provides a readily available supply of proline to the fibroblasts resulting in collagen synthesis. Indeed IL-4Rα LysCre mice, which are deficient in IL-4Rα specifically on macrophages and neutrophils, do not develop M2 macrophages and following *S. mansoni* infections there is endotoxemia and mortality of all infected mice (Herbert et al., 2004). It should be noted, that seminal studies showing a role for IL-13 in *S. mansoni* egg granulomatous fibrosis led to the evaluation of IL-13 as a therapeutic target in other fibrotic conditions such as asthma (Kraft, 2011; Wynn and Ramalingam, 2012). In addition to roles for cytokines chemokines are implicated in granuloma formation (Chiu et al., 2003). Chemokines such as CCL2, CCL3, CCL4, CCL7, CCL11, CCL12, and chemokine receptors CCR1, CCR2, CCR3, and CCR4 have all been shown to be associated with an exacerbated disease in animal studies and have been found at higher levels in the plasma or serum of schistosomiasis patients (Souza et al., 2008).

S. MANSONI EGGS AND ASSOCIATED HEPATOTOXICITY

Whilst the egg granuloma is detrimental due to the inflammation and associated fibrosis, the formation of the granuloma is essential in protecting the host from the toxins secreted by the egg. It must be stressed that in mice the development of hepatotoxicity is unique to *S. mansoni* infections, and is not seen in *S. haematobium* or *S. japonicum* (Fallon, 2000). Antigens secreted by schistosome eggs are potent inducers of the immune system and some are hepatotoxic, and if these antigens are not sequestered or neutralized the resulting inflammatory response can cause lasting damage to the host tissue (Dunne et al., 1981, 1991). As discussed above, along with T cell-dependent antibodies, the granulomatous lesions act to prevent these toxins reaching the hepatocytes (**Figure 1**).

Many aspects of the egg-induced immune response are mediated by glycosylated SEA (Harn et al., 1989; Hokke and Yazdanbakhsh, 2005). SEA glycoproteins collectively display a very complex set of glycans, comprising both specific schistosome glycans and molecules expressed in the mammalian host also (Jang-Lee et al., 2007). The ability of *S. mansoni* eggs to induce Th2 differentiation during infection is underscored by the observation that eggs-alone, or SEA released by the eggs through pores in the shell, is sufficient to drive Th2 polarization in naïve uninfected mice (Jankovic et al., 2004). A portion of SEA components are excreted by the schistosome egg forming the excretory/secretory (ES) fraction, while others come into contact with the host after eggs die and release their soluble contents into the surrounding tissue. Proteomic studies have shown that over a 1000 proteins can be detected in SEA, with a broad range of functions on targets located both inside (cytosolic and nuclear proteins) and outside (membrane proteins, secretory proteins) the cell (Ashton et al., 2001; Mathieson and Wilson, 2011). Distinctive glycan elements abundantly present on SEA and ES glycoconjugates are recognized by PRRs (Guo et al., 2004; Saunders et al., 2009; Ritter et al., 2010). One well characterized *Schistosoma* egg glycan is the Lewis(X)-containing lacto-*N*-fucopentaose III (LNFPIII), which has potent immunomodulatory activity (Bhargava et al., 2012; Tundup et al., 2012). In the context of defective granuloma formation leading to microvesicular steatosis during infection (**Figures 1** and **2**), it is interesting that LNFPIII has recently been shown to suppress liver lipogenesis and protects against hepatosteatosis (Bhargava et al., 2012; Tundup et al., 2012).

The most characterized egg secretions are derived from the highly cationic egg fraction (CEF6) of the SEA containing two important antigens, namely omega-1 (ω-1) and alpha-1 [α-1; more recently termed IPSE (IL-4-inducing principle of *S. mansoni* eggs) or *S. mansoni* chemokine binding protein (SmCKBP)]. Dunne et al. (1981) firstly formally characterized ω-1 and α-1. Immunochemical characterization of ω-1 using sera from mice and humans infected with different schistosome species clarified this antigen as specific to *S. mansoni* (Dunne et al., 1991). ω-1 is a 31 kDa monomeric glycoprotein, with a potent T2 ribonuclease

(RNase) activity (Steinfelder et al., 2009) and is associated with significant hepatotoxicity (Fitzsimmons et al., 2005). In addition, transfer of antisera against ω-1 prevents hepatocyte damage in *S. mansoni*-infected T cell depleted mice confirming the hepatotoxic effects of ω-1 (Dunne et al., 1991). Recently, it has been hypothesized that ω-1 conditions mouse DCs to promote Th2 differentiation *via* a mechanism involving mannose receptors, which appear crucial for the efficient recognition and internalization of ω-1 by DCs (Everts et al., 2012). Importantly, after translocation into the cytosol, ω-1 programs DCs to drive a Th2 polarization in an RNase-dependent manner by interfering with ribosomal function and protein synthesis (Everts et al., 2012).

α-1 consists of two immunologically cross-reactive 41 and 36 kDa dimers, each of which consists of one unique and one common glycoprotein subcomponent (Dunne et al., 1991). It is particularly abundant in the sub-shell area of *S. mansoni* eggs from where it is secreted into the surrounding tissue (Schramm et al., 2003). α-1 binds Ig s and activates basophils, leading to histamine release and facilitating the production of Th2 cytokines, in particular IL-4 (Schramm et al., 2007). It has been also demonstrated that α-1 contains a functional C-terminal nuclear sequence that binds DNA leading to a potential alteration in the gene expression of the host cell (Kaur et al., 2011).

An immunomodulatory egg-antigen was identified during a screen for CKBPs in antigen extracts from *S. mansoni*. An SmCKBP was identified in SEA as a 36 kDa protein specifically secreted by live *S. mansoni* eggs (Smith et al., 2005). Binding assays showed that this egg-antigen specifically binds chemokines, such as CXCL8, CCL3, and CX_3CL1, CCL2, and CCL5. The secretion of a CKBP by live schistosome eggs within the granuloma, suggests that this antigen may block certain chemokines to facilitate granuloma formation and alter the cellularity of the granuloma (Smith et al., 2005). As SmCKBP and α-1 are the same glycoprotein, it is an example of a molecule that may orchestrate both the cellular content and activation state of cells within the granuloma.

Collectively and paradoxically, while the eggs are the cause of pathology during *S. mansoni* infection, they also afford some protection, acting to minimize collateral tissue damage in both the liver and intestine. Therefore, even though the progression of a granuloma into a fibrotic lesion can lead to death from portal hypertension and hemorrhaging, ironically in the absence of a granulomatous response, hepatic murine schistosomiasis results in a more acute and lethal disease.

CONCLUSION

Although egg-induced granulomas are detrimental to the infected host, it is clear that the inflammatory response also fulfills an important host-protective function during *S. mansoni* infection. The granuloma, rich in cells such as Th2 cells, eosinophils, and M2 macrophages, acts to protect the surrounding host tissue from the toxins released by the egg, not only by providing a physical barrier between the egg and the tissue, but also by sequestering the antigenic products secreted by the egg. This is of vital importance, particularly in the liver due to the hepatotoxicity associated with egg-antigens. However, the constant activation of the immune system over time, in particular the type 2 immune responses, results in excessive "wound healing" and invariably leads to the development of fibrotic lesions in the place of the granulomas.

While egg-associated granuloma formation clearly benefits the host, it is also associated with pathology in infected individuals. Conversely, the parasite uses the granuloma to facilitate excretion of its eggs, without killing the host, to continue the life cycle. The granuloma is thus both friend and foe during infection.

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Granuloma formation in pulmonary sarcoidosis

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Sarcoidosis is a granulomatous disorder of unknown cause, affecting multiple organs, but mainly the lungs. The exact order of immunological events remains obscure. Reviewing current literature, combined with careful clinical observations, we propose a model for granuloma formation in pulmonary sarcoidosis. A tight collaboration between macrophages, dendritic cells, and lymphocyte subsets, initiates the first steps toward granuloma formation, orchestrated by cytokines and chemokines. In a substantial part of pulmonary sarcoidosis patients, granuloma formation becomes an on-going process, leading to debilitating disease, and sometimes death. The immunological response, determining granuloma sustainment is not well understood. An impaired immunosuppressive function of regulatory T cells has been suggested to contribute to the exaggerated response. Interestingly, therapeutical agents commonly used in sarcoidosis, such as glucocorticosteroids and anti-TNF agents, interfere with granuloma integrity and restore the immune homeostasis in autoimmune disorders. Increasing insight into their mechanisms of action may contribute to the search for new therapeutical targets in pulmonary sarcoidosis.

Keywords: pulmonary sarcoidosis, granuloma, formation, integrity, dendritic cells, T helper 1 cells, T helper 17 cells, regulatory T cells

INTRODUCTION

Sarcoidosis is a granulomatous disorder of unknown cause, affecting multiple organs, but mainly the lungs. In 10–30% of the cases, sarcoidosis becomes chronic and progressive leading to debilitating disease and sometimes death (1). Its etiology is intriguing, since a part of its definition (i.e., *unknown cause*) makes it uniquely different from granulomatous disorders arising from exposure to a known chronically persisting antigen, such as tuberculosis, visceral leishmaniasis, and chronic beryllium disease (2, 3). Nevertheless, several observations support an antigen-induced disease etiology. First, epidemiological research identified environmental and occupational risk factors, such as exposure to musty odors and insecticides (4). Second, infectious agents, including *Propionibacterium acnes* (*P. acnes*) and *Mycobacterium tuberculosis* (*Mtb*), have been implicated, since genomes of these species are detected within sarcoid granulomas (5). A role for mycobacterial peptides is further supported by the presence of T lymphocytes that are highly responsive toward 6-kDa early secreted antigenic protein (ESAT-6) or catalase peroxidase (KatG) in the broncho-alveolar lavage fluid (BALF) of sarcoidosis patients (6–8). Third, a limited clonality of $CD4^+$ T cells, expressing the AV2S3 T cell receptor, was demonstrated within the lungs of HLA-DRB1*03 positive sarcoidosis patients, which is consistent with an antigenic response (9–12). Finally, evidence for an antigen-induced disease lies within the granulomatous reaction that is virtually indistinguishable from sarcoid granulomas and occurs in individuals with sarcoidosis upon subcutaneous injection of homogenates from allogeneic sarcoid spleen or lymph nodes (LNs), i.e., the Kveim–Siltzbach test (13, 14).

GENETIC RISK FACTORS IN SARCOIDOSIS

People all over the world suffer from sarcoidosis (15). Familial clustering (16), increased concordance in monozygotic twins (17) and variations in susceptibility and disease presentation among different ethnic groups (18), suggest the importance of genetic, next to environmental risk factors in the etiology.

Genome-wide association studies (GWAS) identified polymorphisms within genes coding for proteins involved in T cell activation, differentiation, proliferation, and survival, including NOTCH 4 and ANXA11 (19, 20). Additionally, GWAS and case-control studies identified important genetic risk factors within the antigen presentation locus at 6p21.3, which contains genes encoding proteins involved in both antigen presentation and T cell regulation, including human leukocyte antigen (HLA) and *butyrophilin-like* protein (BTNL)-2, respectively (20–23).

Specific HLA class II antigens are associated with certain sarcoidosis disease phenotypes. For example, the HLA-DRB1*03 and DQB1*0201 alleles have been associated with an acute disease onset, Löfgren syndrome and resolving disease, whereas in contrast HLA-DRB1*15 and DQB1*0601 are associated with chronic sarcoidosis (24–27). It is conceivable that both resolving and persistent sarcoidosis arise due to a unique combination of a specific genetic background and exposure to one or several environmental triggers (28). This unique combination might lead to persistent stimulation of the immune system, contributing to granuloma formation and sustainment.

In this article we review the current knowledge on the role of the immune activation in pulmonary sarcoidosis and propose a hypothesis on the origin of granuloma formation. Secondly, we

aim to discuss granuloma integrity, highlighting areas for research into new therapeutical targets.

GRANULOMA FORMATION

A well-developed sarcoid granuloma consists of a tightly formed conglomerate of epithelioid- and multinucleated-giant cells (MGCs) encircled by lymphocytes, especially $CD4^+$ T helper (Th) cells, but also rare $CD8^+$ T cells and B cells (1). Both granuloma formation and integrity depend on the availability and supply of these different cells (29). The chronological order of immunological events and the exact role of these cells during the sarcoid granulomatous response remain obscure, due to the lack of an animal model for sarcoidosis. Nevertheless, careful clinical observations and in-depth research on functional properties of different cells involved provide essential information to unravel the cellular and molecular mechanisms of granuloma formation.

CLINICAL SIGNS

Cardinal features of pulmonary sarcoidosis are mediastinal lymphadenopathy, parenchymal, and airway granulomas, giving rise to upper lobe nodules in a perilymphatic or bronchovascular distribution and signs of a $CD4^+$ T cell alveolitis. An interstitial pneumonitis, found on open lung biopsy, is classically thought to represent a very early stage of granuloma formation (30). Spontaneous remission and reactivation of sarcoidosis makes it difficult to ascertain the exact sequence of these cardinal features, however several findings strongly suggest a certain order in the majority of patients, which may add to the hypothesis on granuloma formation as described below.

Although it is well known that patients do not go through all disease stages as described by Scadding (from I to IV) sequentially, arguably pulmonary sarcoidosis starts in the draining LN. As stage I (bihilar lymphadenopathy) is most often asymptomatic, it is conceivable that it precedes pulmonary involvement, seen in stage II and III. Additionally, progression of stage I to II disease is well known, while development of stage I after stage III is uncommon. Finally, a recent trial found an increased diagnostic sensitivity of LN-derived fine needle aspirates, compared with transbronchial lung biopsies (31). These data suggest that the first granulomas are formed within the mediastinal LN, only later followed by granuloma formation within the lungs.

Consequently, LN-specific immune reactions are important in early sarcoid granuloma formation, such as antigen presentation by dendritic cells (DCs). DCs are the only cells capable to pick up antigens and migrate to the LN where they present antigens to naïve T cells. Hereby they initiate highly specific clonal T cell differentiation and proliferation (32). Alternatively, LN-resident DCs may encounter antigenic particles, which we propose are submicroscopic and may therefore have passively migrated through the afferent lymph. The activated and differentiated Th cells migrate toward the site of inflammation, orchestrated by chemokines.

Macrophages contribute to early recognition of the putative sarcoid antigen in the lungs, thereby attracting mononuclear cells, including monocytes and LN-activated lymphocytes. The ensuing influx of cells leads to an interstitial pneumonitis, characterized by a mixed mononuclear cell infiltrate in the alveolar wall and $CD4^+$ T cell alveolitis (30).

At the site of antigen encounter, antigen-presenting cells (APCs) induce persistent stimulation of the immune response, mediated by HLA-related proteins, leading to continuous recruitment and local expansion of lymphocytes and eventually granuloma formation. The central localization of macrophages within the final epithelioid aggregate supports an important role in antigen presentation at the site of granuloma formation. Alternatively, DCs may play a critical role in antigen presentation within the granuloma. Their capacity for antigen sampling within the lymph fluid makes them likely candidates to contribute to the induction of the perilymphatic localized granulomas (33, 34).

In the following paragraphs we describe the current knowledge on the role of macrophages, DCs, and lymphocytes in sarcoid granuloma formation in more detail, also summarized in **Figure 1**.

MACROPHAGES

Upon activation, macrophages release nuclear factor (NF)-κB-dependent pro-inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) (35). In sarcoidosis, BALF cells and monocytes highly express toll-like receptor (TLR)-2 (36, 37) and produce increased amounts of TNF-α, IL-1β, and IL-6 compared with controls, when stimulated with TLR-2 ligands, including ESAT-6 and KatG (7, 36–38). A role for TLR-2 in immune activation and granuloma formation in sarcoidosis is further supported by genetic and mouse studies (38, 39). Lately, continuous TLR-2 ligation by macrophage-derived serum amyloid A has been suggested to contribute to persistent stimulation of the immune response in sarcoidosis (37).

Intrinsically, unstimulated sarcoid-derived alveolar macrophages (AMs) produce increased amounts of IL-1 and TNF-α (40–43) and are highly activated (44, 45). The amounts of spontaneously produced TNF-α by BALF cells *in vitro* correlate with the presence of aggregates of AMs in the tissue (46, 47). Only AMs from patients with active and progressive disease produce increased amounts of TNF-α (48–51). These data highlight the role of TNF-α in granuloma formation and integrity, also supported by mouse studies (52–54).

Important mechanisms of action of TNF-α include macrophage activation, promotion of cellular migration toward the site of inflammation and leukocyte adhesion (52, 55, 56). In a mycobacterial-driven mouse model, TNF-α is responsible for the early production of chemokines that attract mononuclear cells to the site of inflammation, such as RANTES, MIP-1α, MIP-1β, MIP-2, and MCP-1 (55), of which increased amounts are found in sarcoidosis BALF (57–59). In active sarcoidosis, AMs produce high amounts of CCL20, when stimulated by TNF-α and IL-1β (60). CCL20 is a chemokine with high affinity for chemokine receptor CCR6, therefore attracting DCs, B cells, and specific T cell subsets toward the lungs (60, 61). Similarly, AM-derived CXCL10 and CXCL16 contribute to $CXCR3^+$ and $CXCR6^+$ $CD4^+$ Th cell recruitment (62, 63).

In a mycobacterial-driven granuloma model, efficient cellular recruitment, mediated by AM-derived CXCL10 and CXCL16, depends on interferon-γ (INF-γ) (53). During the early innate response natural-killer (NK) cells are important producers of INF-γ, when stimulated by TNF-α, IL-1, and IL-12. In sarcoidosis, the size of a distinct NK cell subpopulation

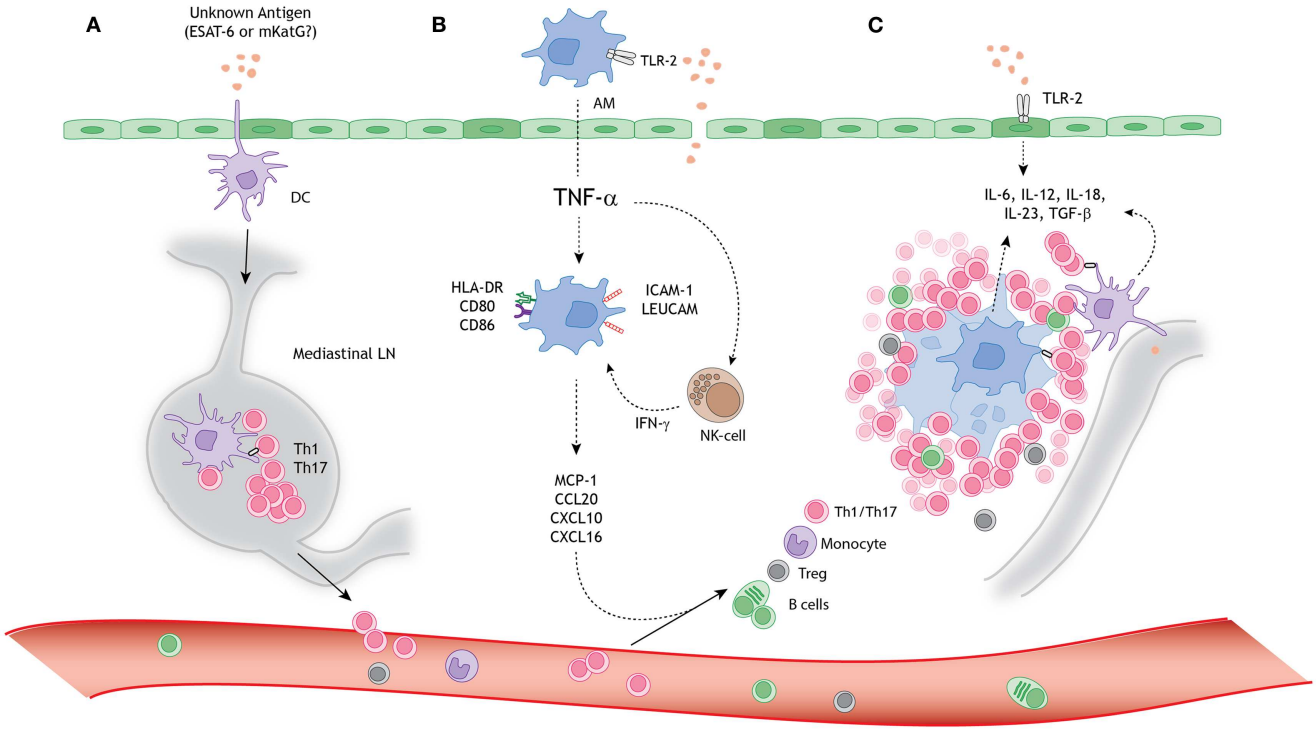

FIGURE 1 | A schematic model for granuloma formation in pulmonary sarcoidosis. An unknown airborne-antigen activates **(A)** interstitial dendritic cells (DCs), **(B)** alveolar macrophages (AMs), and **(C)** alveolar epithelial cells type II (AEC-II) (*dark green*), simultaneously. This process is initiated by toll-like receptor-2 (TLR-2) ligands, possibly *Mycobacterium tuberculosis*-derived ESAT-6 or mKatG. **(A)** The interstitial DCs pick up the putative antigen and migrate toward the mediastinal lymph nodes (LNs), where they initiate differentiation and clonal expansion of T helper (Th)1 and 17 cells. **(B)** Simultaneously, AMs produce tumor necrosis factor-α (TNF-α), which initiates upregulation of activation (HLA-DR and CD80/86) and adhesion (ICAM-1 and LeuCAM) molecules. Macrophages produce chemokine ligands (MCP-1, CCL20, CXCL10, and CXCL16) under stimulation of both TNF-α and natural-killer (NK) cell-derived interferon-γ (INF-γ), thereby attracting Th1/17 cells, monocytes, regulatory T cells (Tregs), and B cells. **(C)** The lung environment is characterized by the presence of Th1 and Th17 favoring cytokines, such as IL-6, IL-12, IL-18, IL-23, and TGF-β, produced by macrophages, perilymphatic DCs, and AEC-II. Persistent stimulation, mediated by antigen presenting cells (APCs), leads to continuous cellular recruitment to the site of inflammation, which leads to granuloma formation. Tregs infiltrating the granuloma fail to diminish the exaggerated immune response, thereby contributing to granuloma persistence and integrity.

($CD56^{bright}CD94^{high}KIR^{low}$) is increased in the BALF compared with controls (64). Furthermore, higher proportions of NK cells were found to correlate with a poor outcome (65).

Once recruited, TNF-α is needed for leukocyte adhesion, since an abrogation of tightly formed granulomas in TNF-α-deficient mice is observed following mycobacterial infection (55). In sarcoidosis, TNF-α induced the expression of intracellular adhesion molecule-1 (ICAM-1) on AMs, leading to cellular aggregation (66). Additionally, leukocyte adhesion molecule (LeuCAM) expression, such as CD11a/b/c and CD18 (67), is increased in sarcoid AMs compared with controls.

Following adhesion, epithelioid histiocytes and monocyte-derived DCs (moDCs) can fuse to MGCs when stimulated by local cytokines, such as TNF-α, GM-CSF, IL-17A, CCL20, and INF-γ (68, 69). Patient-derived macrophages and monocytes show an enhanced potential to form MGCs *in vitro*, compared with healthy controls and other granulomatous diseases (70).

Importantly, sarcoid-derived AMs have an increased accessory function on autologous blood- and lung-derived T lymphocytes, when compared with controls (71–73). Macrophages are not capable to migrate to the LN to induce naive T cell activation, making them weak APCs. Nonetheless, in sarcoidosis, macrophages might contribute to local antigen presentation, enhancing proliferation of chemokine-recruited memory Th cells.

In summary, macrophages are important for the initial accumulation, aggregation, and fusion of the cellular building blocks needed for granuloma formation. This process is mediated by the strong immune modulatory capacities of TNF-α and assisted by NK cells, which produce INF-γ.

DENDRITIC CELLS

Only a few studies investigated the role of DCs in sarcoid granuloma formation (47, 74). Our group has shown that granuloma formation surrounding intravenously injected antigen-loaded beads trapped in the lung vasculature is dependent on DC-initiated Th cell proliferation within the mediastinal LN (75). In sarcoidosis, an accumulation of mature ($Fascin^{+}HLA\text{-}DR^{+}DC\text{-}LAMP^{+}$) DCs is found surrounding LN granulomas, adjacent to $CD3^{+}$ lymphocytes, suggesting DC-T cell interaction at this site (76). Mature ($CD11c^{+}CD86^{+}$) DCs are found surrounding

granulomas in sarcoid-derived mucosal biopsies (77), further supporting a role for DCs in airway and parenchymal granuloma formation.

An impaired accessory function of *ex vivo* blood-derived myeloid DCs (mDCs) has been suggested to contribute to granuloma formation, as clearance of the putative antigen may be ineffective and the immune system turns to granuloma formation as a default immunological response (78). In contrast, our group isolated BALF mDCs of sarcoidosis patients and found them to be immunocompetent, initiating proliferation of allogeneic, naïve T lymphocytes comparable with mDCs from healthy controls (77). Similarly, *in vitro* cultured moDCs showed a comparable accessory capacity as controls, although they are intrinsically prone to produce TNF-α (77, 79). Hence, it is most likely that DCs are involved in granuloma formation, instead of displaying diminished antigen-presenting capacities.

Differentiation of T lymphocytes depends on the local cytokines surrounding the initiating APC (80). Although it is very likely that LN-specific interactions, mediated by DCs, are responsible for the initial T cell polarization toward a Th1 and Th17 phenotype as observed in sarcoidosis, direct evidence is still lacking.

LYMPHOCYTES

Sarcoidosis is characterized as a Th1- (81) and more recently a Th17-mediated disease (61, 82), based on the accumulation of INF-γ, IL-2, and IL17-producing Th cells in the lungs of patients with active sarcoidosis (44, 61, 82–84).

Th1 differentiation depends on IL-12 and IL-18, which are increased in BALF of sarcoidosis patients (85, 86). Alveolar epithelial cells type II (AEC-II) may contribute to this Th1-favoring environment, since patient-derived AEC-II produce IL-18 upon TLR-2 stimulation (87, 88). Additionally, AEC-II may contribute to $CXCR3^+$ Th1 cell recruitment by production of CXCL10 (89).

Th17 differentiation is driven by IL-6 and TGF-β, both produced by sarcoid-derived BALF cells (90, 91), whereas survival and proliferation of this subset is IL-23-dependent (92–94). Increased expression of the IL-23-receptor and IL-17, both expressed by Th17 cells, is found in blood-, lung-, and LN-derived lymphocytes of active sarcoidosis patients, and not in inactive disease (61, 82). Recently, ESAT-6-specific Th17 cells in the BALF of sarcoidosis patients were found (95). Additionally, IL-17A is essential for granuloma formation in the lung during mycobacterial infection (96) or in chronic granulomatous disease (97).

We recently found that the proportions of circulating IL-17A/IFN-γ and IL-17A/IL-4 double-producing cells are significantly increased in the peripheral blood of patients and are present in substantial numbers in BALF (82). Findings in several autoimmune diseases have indicated the pathogenic potential of $CD4^+$ Th cells producing both IL-17 and IFN-γ (98, 99). Processes underlying Th17 cell induction in sarcoidosis remain obscure, but the presence of these cells can suggest a role for autoimmune responses in sarcoidosis. B lymphocytes and plasma cells are found surrounding sarcoid granulomas (100). Additionally, active sarcoidosis patients have increased serum levels of B-cell-activating factor (BAFF) (101). Since B cell maturation and function depends on BAFF, its aberrant expression can initiate defective selection of autoreactive B cells, leading to autoantibody production (101, 102). In sarcoidosis, approximately 30–60% of the patients exhibit antinuclear antibody (ANA) positivity (101, 103).

A SNP in the IL-23 receptor gene has been associated with chronic sarcoidosis (104), which may contribute to Th17 cell development in sarcoidosis. Since IL-23 is a heterodimer of the p19 subunit and the p40 subunit of IL-12 (105) the Th1- and Th17-promoting cytokines share a common therapeutical target. Ustekinumab, a neutralizing antibody against the IL-12/IL-23 p40, was shown to be successful in the Th1/Th17-mediated diseases psoriasis and Crohn's disease (CD) (106, 107), but not in chronic pulmonary or skin sarcoidosis (108, 109).

GRANULOMA INTEGRITY

In the majority of the sarcoidosis patients, granulomas spontaneous resolve within several years, without need for therapy. However, a substantial proportion of the patients develop chronic progressive disease, whereby granulomas persist and form fibrotic lesions, leading to debilitating disease, and sometimes death (1, 110). The immunological response, determining granuloma sustainment is not well understood.

REGULATORY T CELLS

Regulatory T cells (Tregs) play an important role in diminishing Th cell specific responses and are pivotal for maintenance of self-tolerance and immune homeostasis (111). An impaired immunosuppressive function of sarcoid-derived Tregs has been suggested to contribute to the on-going, exaggerated immune response, since sarcoid blood-derived ($CD4^+CD25^{high}$) Tregs fail to inhibit granuloma growth in an *in vitro* granuloma culture model (112, 113). Subsequently, an impaired immunosuppressive function of both blood- and BALF-derived sarcoidosis Tregs has repetitively been described on autologous and allogeneic healthy Th cell proliferation (114–116). These studies also show that sarcoid-derived Tregs fail to inhibit production of TNF-α, INF-γ, and IL-2, contributing to granuloma formation, rather than diminishing the immune response (112, 113, 116). It remains unknown what mechanism(s) underlies this impaired function.

Active and persisting sarcoidosis was recently associated with a global $CD4^+$ T cell subset dysfunction (116). Notably, both Th anergy and Treg malfunctioning were restored in patients with disease resolution (116). These results highlight the complex interplay between pro-inflammatory and anti-inflammatory responses needed for granuloma integrity. This fine balance may explain contradictory results with regard to reported Treg numbers in the BALF (112–119) (**Table 1**). Low BALF Tregs (i.e., less immunosuppression) in patients have been associated with a favorable prognosis in a Scandinavian population (118). In contrast, a German study reported decreased BALF Treg numbers in sarcoidosis patients who develop chronic (active) disease, when compared with controls and patients who develop spontaneous resolution (115). Similarly, CD1d-restricted natural-killer T (NKT) cells with immunoregulatory function are greatly reduced in the peripheral blood of all sarcoidosis patients, except Löfgren patients (120).

Table 1 | An overview of studies reporting regulatory T cell (Treg) proportions and functional properties in pulmonary sarcoidosis.

Study	Methods			Proportions			Function			Remarks
	Population	Treg definition	Technique	Blood	BALF	LN	Blood	BALF	LN	
Miyara et al. (112)	Active disease	$CD4^+CD25^+$ % of $CD4^+$	FC/IHC	↑	↑[b]	↑	↓[^]	=[b]	=	^Blood-derived Tregs reduce autologous T cell proliferation similarly as controls, but do not inhibit the release of TNF-alpha and INF-γ
Idali et al. (117)	Active disease	$CD4^+FoxP3^+$ % of $CD4^+$	FC/PCR	↓	↓					BALF Treg proportions are significantly higher than blood Treg proportions in both healthy controls and patients
Taflin et al. (113)	Active disease	$CD4^+CD45RA^-FoxP3^{++}$ % of $CD4^+$	FC/IHC	↑		↑[b]	↓			$FoxP3^+$ Tregs in the sarcoid LN are highly proliferative ($Ki67^+$)
Prasse et al. (115)	Pre-treatment patients	$CD4^+CD25^+CD127^-$ % of $CD4^+$	FC		↓[^]			↓[c,#]		^BALF Treg proportions are decreased in patients who develop active chronic disease, defined after 1 year follow-up #Vasoactive intestinal peptide (VIP) inhalation increased the number of BALF Tregs and the immunosuppressive function
Rappl et al. (114)	Unknown	$CD25^+CD7^-$% of $CD4^+$ $CD45RO^+$ $FoxP3^+CD127^-$	FC		↑		↓			Increased proportions of $CD4^+$ $FoxP3^+$ $CD127^-$ Tregs are CD7-, compared with healthy controls
Wiken et al. (118)	Active disease[a]	$FoxP3^+$ % of $CD4^+CD45RO^+$ $CD27^+$	FC		↓[d]					BALF Tregs proportions are siginificantly decreased in HLA-DRB1*0301 positive patients, which are mostly (82%) Lofgren patients
Darlington et al. (119)	Active disease	$CD4^+FoxP3^+$ % of $CD4^+$	FC			=[b]				% FoxP3 expressing $CD4^+$ T cells is inversely correlated with % T cells with AV2S3 > 10% in BALF
Oswald-Richter et al. (116)	Active disease	$CD4^+CD45RO^+$ $CD25^{high}$ % of $CD4^+$	FC	↑			↓			Treg malfunctioning restored during disease resolution

All results are compared with healthy controls, unless specified otherwise. Flowcytometry (FC), Immunohistochemistry (IHC), Polymerase chain reaction (PCR), broncho-alveolar lavage fluid (BALF), lymph node (LN).

[a] *HLA-DRB1*0301 positive sarcoidosis patients were analyses vs. HLA-DRB1*0301 negative sarcoidosis patients.*

[b] *Compared with diseased controls.*

[c] *Compared with post-treatment.*

[d] *Compared with HLA-DRB1*0301 negative patients.*

Taken together, these studies imply different roles for immune regulatory cells in sarcoidosis, either contributing to or preventing an on-going, exaggerated immune response. Arguably, whereas in the early sarcoid response there may be no need for Tregs to inhibit an effective immune response, during persistent stimulation immune regulatory cells should function as a natural brake on the exaggerated response to prevent immunopathology and autoimmunity.

INTERFERING WITH GRANULOMA INTEGRITY

Effective treatment agents used for sarcoidosis interfere with granuloma integrity and would ideally prevent fibrogenesis. Glucocorticosteroids (GCs), the main stay of sarcoidosis therapy, partially exert their beneficial effect by repression of NF-κB-related cytokine gene transcription and induction of lymphocyte apoptosis (121, 122). Using a mouse model, Tregs are found to be less sensitive to GC-induced apoptosis compared with Th cells, favoring an anti-inflammatory milieu (123, 124). Similarly, anti-TNF agents induce monocyte and lymphocyte apoptosis (125–127), while improving Treg numbers (123). Interestingly, infliximab, which blocks membrane-bound TNF-α, is uniquely associated with a high risk of reactivation of latent *Mtb* infection, whereas etanercept, solely blocking secreted TNF-α, is not (29). This phenomenon implies a critical role of membrane-bound TNF-α signaling in granuloma integrity (29), which is further supported by mouse studies (128).

Whether GCs and anti-TNF agents interfere with the delicate Th/Treg balance in pulmonary sarcoidosis, remains to be elucidated. Research into this field will shed more light on the role of Tregs in sarcoid pathology and whether Treg induction holds a promising new therapeutical strategy. Finally, an interplay between anergic Th cells, IL-10, alternatively activated macrophages (M2), CCL18 and lung fibroblasts has recently been suggested to contribute to fibrotic remodelling of the lung in chronic sarcoidosis (129). These insights yield new therapeutical targets to prevent irreversible organ damage in chronic pulmonary sarcoidosis patients.

CONCLUSION

Sarcoidosis is an intriguingly complex granulomatous disorder, characterized by an exaggerated Th1/17 immune response, initiated by APCs, and maintained due to malfunctioning of Tregs. Refining insight into immunological events that determine granuloma fate may help identify new therapeutical targets and patients who will benefit such therapy in the future.

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