EMERGING CONCEPTS OF INNATE IMMUNE RESPONSES TO NEGLECTED TROPICAL DISEASES

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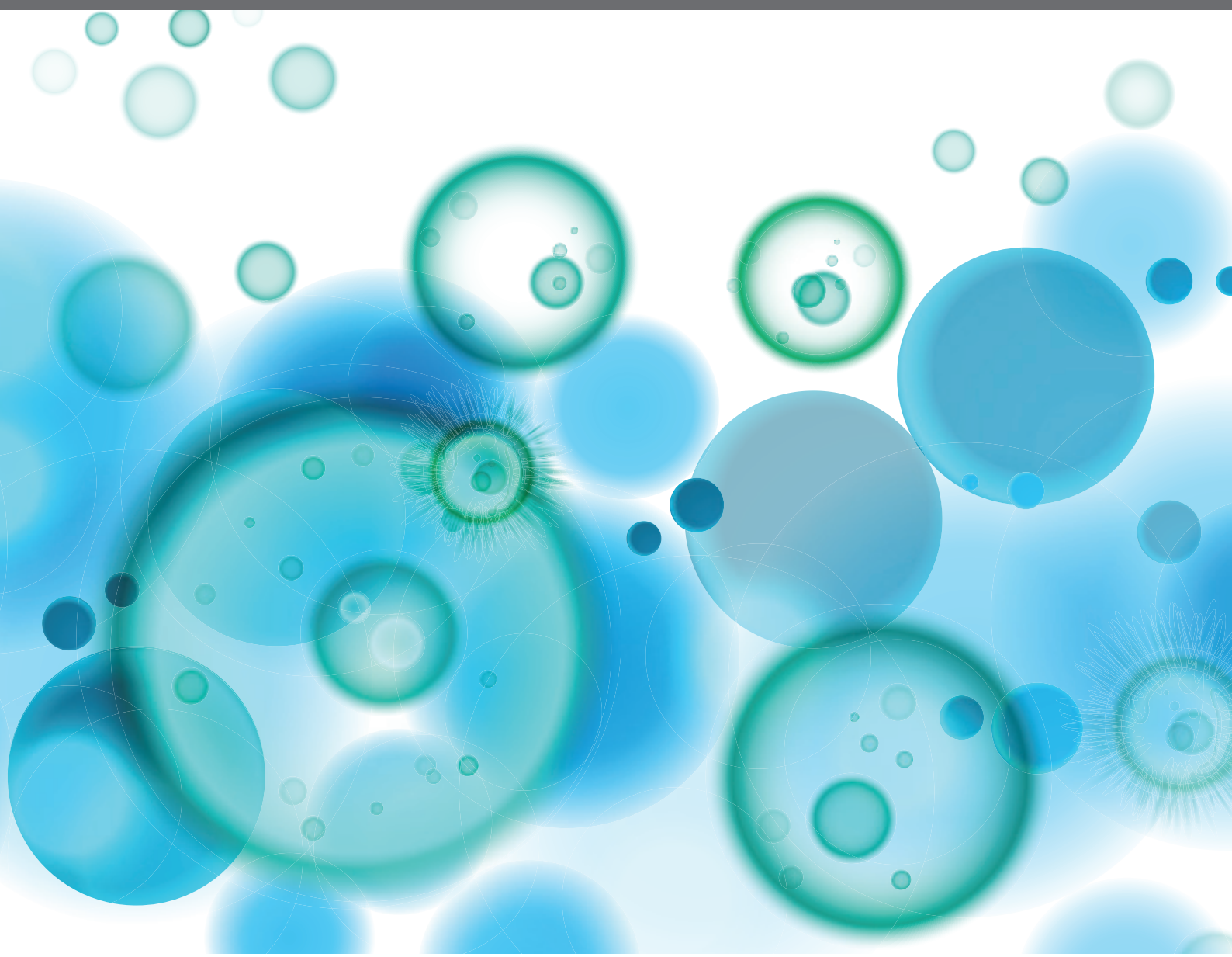

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EMERGING CONCEPTS OF INNATE IMMUNE RESPONSES TO NEGLECTED TROPICAL DISEASES

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EDITORIAL
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Editorial: Emerging Concepts of Innate Immune Responses to Neglected Tropical Diseases

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Editorial on the Research Topic

Emerging Concepts of Innate Immune Responses to Neglected Tropical Diseases

Neglected tropical diseases (NTDs), a collective of infectious diseases predominantly reported in under-resourced settings, directly affect over a billion people. Multiple initiatives have been launched to combat NTDs but they continue to place a significant economic and social burden on developing nations. Beyond their immediate importance, studies of NTDs have become important pillars of immunology: clinical observation, and experimental animal models, of NTDs helped define the Th1/2 paradigm (1–3). This Research Topic, presented as original research and review articles, focused on recent advances in our understanding of innate immune mechanisms and cell subsets, and of how innate cells can influence the outcome of NTDs.

Van Bockstal et al. demonstrated that type I interferons exacerbate *Leishmania* infections associated with visceral disease. Enhanced macrophage susceptibility to *Leishmania infantum* and *L. donovani* following incubation with IFN-α was linked to upregulated sialoadhesin (Siglec-1/CD169, Sn) surface expression and in a series of experiments involving Sn-deficiency or blockade, susceptibility was restored to normal levels. These data raise the possibility that targeting of Sn in early *Leishmania* infection could limit progression to visceral disease.

Lecoeur et al. applied an intricate flow cytometry-based procedure to demonstrate that *Leishmania* subverts the transcription factor landscape by escaping the Toll-like receptor (TLR)–NF-kB–NLRP3 axis and stalling maturation. Transcriptomic analyses of *Leishmania*-infected DC revealed that DC infected by non-opsonized parasites maintained an immature phenotype that was associated with down-regulation of genes related to pro-inflammatory TLR signaling while infection of DC with opsonized parasites enhanced this profile, with DC displaying a semi-mature phenotype. The authors reasonably speculate that this *Leishmania*-specific signature could have relevance to infection with other intracellular pathogens.

Although myeloid innate immune cells are the dominant sensors of microbes, Stögerer and Stäger highlight that B and T cells also express PRRs like TLRs. TLR signaling, in particular MyD88-dependent TLRs and endosomal TLR7 and TLR9, is involved in autoreactive B cell activation and in visceral leishmaniasis is proposed to cause hypergammaglobulinemia and disease exacerbation. DAMPs can trigger TLR7 and induce cell death in T cells during chronic infection of *Leishmania donovani*. It is thus important to consider the effect of TLR agonists on T and B cells, and not only on myeloid cells, when developing new vaccination strategies, particularly for therapeutic purposes.

The disruption of splenic white pulp is accompanied by disease progression in mammals that are susceptible to fatal visceral leishmaniasis. De Melo et al. document morphological remodeling of splenic compartments following experimental *L. infantum* infection. One month after parasite inoculation of BALB/c mice a decline in the number of plasmacytoid DC was observed along with

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hyperplasia in the white pulp. By two months, splenomegaly remained and was now associated with increased numbers of macrophages, B and T cells and plasma cells. Unlike the organized distribution of lymphoid tissue inducer (LTi) cells around the periarteriolar lymphoid sheath observed in control mice, LTi were increased and scattered throughout the red pulp in *Leishmania*-infected mice. By three months of infection, the relative frequencies of follicular and plasmacytoid DC were increased and splenic IL-6 and IFN-gamma production had commenced.

In their contribution Serrano-Coll et al. speculate that alterations in the skin and peripheral nerves during leprosy are related to activity in the Notch signaling pathway. They observed that *Hes-1* gene expression was downregulated, while *Runx-1* was upregulated, in the skin of leprosy patients. Immunohistochemistry revealed a corresponding reduction of Hes-1 protein levels in the epidermis, eccrine glands and hair follicles. Increased expression of Runx-1 was observed in infiltrating inflammatory cells. The authors reason that these changes could render the infected skin conducive for *M. leprae* survival and proliferation.

While *Mycobacterium leprae* causes leprosy, the vast majority of exposed individuals do not develop disease. Van Hooij et al. assessed the relationship between innate immune markers and *M. leprae* infection by studying individuals at high risk of infection. Similar to leprosy patients, the individuals at risk had higher ApoA1 and S100A12 levels than control subjects. Importantly, higher S100A12 and lower CCL4 levels in response to *M. leprae* were observed among households where *M. leprae* infection and leprosy were not detected beyond the index case, suggesting that these molecules are involved in a response that restricts *M. leprae* dissemination.

Boldt et al. took a unique retrospective approach of comparing leprosy patients who have had/had not HBV infection and addressed associations of genes in the complement pathway with susceptibility to HBV in leprosy patients, demonstrating polymorphisms compromising activation of the lectin pathway of complement, especially ficolin, as modulating the susceptibility. The associations suggest a critical role of the lectin pathway in controlling HBV infection which is maintained and even reinforced within the context of leprosy disease. Thus, residents in NTD-endemic areas can experience altered responses against other immune challenges, posing obvious and significant public health challenges.

While most pathogenic mycobacteria evade innate immune responses to replicate inside host macrophages and induce granulomas that contain but do not eliminate the bacteria, *Mycobacterium ulcerans* has acquired the virulence plasmid pMUM that allows the synthesis of mycolactone, a macrolide toxin that causes the chronic, necrotizing skin lesions that characterize Buruli ulcer. By comparing the pathogenesis of *M. ulcerans* and *M. marinum* diseases, Röltgen and Pluschke summarize current data on innate immune mechanisms against *M. ulcerans* infection. They note that Bacillus Calmette-Guérin (BCG) has limited short-term protective activity against Buruli ulcer and conclude that a more detailed understanding of innate immunity to *M. ulcerans* infection is required to develop an effective vaccine for control in endemic areas.

While the pathogenesis of experimental trypanosome infections has been widely studied after intraperitoneal or intravenous injection, real world infection with African trypanosomes begins when the tsetse fly vector injects the parasites into the dermis during blood feeding. In their review article, Alfituri et al. highlight recent studies of initial infection in the skin. They suggest that by thoroughly defining the mechanisms involved in both establishing African trypanosome infections in the skin and in facilitating their progression through the host, novel approaches to control may be revealed. By assessing *Trypanosoma brucei* infection in lymphotoxin-β-deficient mice (LTβ-/- mice), the group tested their hypothesis that the absence of the skin draining lymph nodes would impede the establishment of infection (Alfituri et al.). To their surprise, LTβ-/- mice exhibited greater susceptibility to *T. brucei* infection, indicating that the early accumulation of the trypanosomes in the skin draining lymph nodes was not essential for systemic infection. Restoration of the microarchitecture of the B cell follicles in the spleens of LTβ-/- mice reduced susceptibility to intradermal *T. brucei* infection and IgG class-switched parasite-specific antibodies became apparent in the circulation. These data suggest that organized splenic microarchitecture is essential for the control of African trypanosomes.

Magez et al. review how innate immune components control salivarian trypanosomes. To avoid their overpopulation and killing of the host, trypanosomes have acquired a system of quorum sensing for density-dependent population growth arrest. The same system could possibly sense infection-associated host tissue damage, in which case the quorum sensing serves to prevent excessive immunopathology. The researchers' own experiments showed that vaccine-induced immune modulation of inflammation, and the CD1d molecule, was central to protection during trypanosome infection. They extend their review by discussing reports indicating that trypanosomes compromise the general host immune system so as to cause significant reductions in the efficacy of multiple non-trypanosome related vaccines (Magez et al.). In a similar vein, Osii et al. reviewed current understanding of how *Plasmodium* infection disrupts DC-T cell interactions to generate T cells that fail to help B cell responses. This not only reduces the production of antibodies necessary to control malaria, but by upregulating negative regulatory molecules *Plasmodium* infection also induces CD4 T cell exhaustion.

Schistosoma parasites enact numerous strategies, including the production of immunomodulatory molecules, alteration of membranes and the formation of granulomas, to promote infection without detrimental exposure to the immune response (Angeles et al.). Keratinocytes, macrophages, DCs and B1 cells produce IL-10 in the skin in response to the cercarial invasion and Sm16, a major molecule from the secreted protein of the cercaria can prevent classical activation macrophage and delay antigen processing. Excretory-secretory products from the cercaria and schistosomula also induce inhibitory molecules such as prostaglandins.

Obata-Ninomiya et al. detail that helminths interact with various epithelial cell surfaces, with the cells subsequently producing a series of type 2 epithelial cytokines that lead to the induction of innate and acquired type 2 immune responses. Increased frequencies of basophils and eosinophils have been documented during helminth infections and in response to nematode parasite re-infection, basophils accumulate in the peripheral tissues for IgE release and secretion of proteases that recruit monocytes, neutrophils and eosinophils. In response to IL-5, eosinophils produce major basic protein to kill the parasites.

By characterizing innate responses in experimental models, and observing them in clinical or veterinary settings, of NTDs, valuable insight is being generated for the development of new control strategies (diagnostics, immune therapy, enhanced vaccination). With the realization of similarities with other infectious and non-infectious diseases, this knowledge can have significant impact beyond NTDs.

AUTHOR CONTRIBUTIONS

MD and YG wrote this editorial. All authors contributed to the article and approved the submitted version.

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REVIEW
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Infections With Extracellular Trypanosomes Require Control by Efficient Innate Immune Mechanisms and Can Result in the Destruction of the Mammalian Humoral Immune System

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Salivarian trypanosomes are extracellular parasites that affect humans, livestock, and game animals around the world. Through co-evolution with the mammalian immune system, trypanosomes have developed defense mechanisms that allow them to thrive in blood, lymphoid vessels, and tissue environments such as the brain, the fat tissue, and testes. Trypanosomes have developed ways to circumvent antibody-mediated killing and block the activation of the lytic arm of the complement pathway. Hence, this makes the innate immune control of the infection a crucial part of the host-parasite interaction, determining infection susceptibility, and parasitemia control. Indeed, trypanosomes use a combination of several independent mechanisms to avoid clearance by the humoral immune system. First, perpetuated antigenic variation of the surface coat allows to escape antibody-mediated elimination. Secondly, when antibodies bind to the coat, they are efficiently transported toward the endocytosis pathway, where they are removed from the coat proteins. Finally, trypanosomes engage in the active destruction of the mammalian humoral immune response. This provides them with a rescue solution in case antigenic variation does not confer total immunological invisibility. Both antigenic variation and B cell destruction pose significant hurdles for the development of anti-trypanosome vaccine strategies. However, developing total immune escape capacity and unlimited growth capabilities within a mammalian host is not beneficial for any parasite, as it will result in the accelerated death of the host itself. Hence, trypanosomes have acquired a system of quorum sensing that results in density-dependent population growth arrest in order to prevent overpopulating the host. The same system could possibly sense the infection-associated host tissue damage resulting from inflammatory innate immune responses, in which case the quorum sensing serves to prevent excessive immunopathology and as such also promotes host survival. In order to put these concepts together, this review summarizes current knowledge on the interaction between

trypanosomes and the mammalian innate immune system, the mechanisms involved in population growth regulation, antigenic variation and the immuno-destructive effect of trypanosomes on the humoral immune system. Vaccine trials and a discussion on the role of innate immune modulation in these trials are discussed at the end.

Keywords: trypanosomes, innate immunity, cytokine, antibody, immunosuppression

INTRODUCTION

Human African Trypanosomosis (HAT) and Animal Trypanosomosis (AT) are parasitic diseases that are caused by unicellular protozoan organisms of the class Kinetoplastida. While classic HAT is a disease that is strictly confined to specific areas of sub-Sahara Africa, AT is a disease that is found on all continents except Antarctica. Most pathogenic livestock trypanosomes are salivarian trypanosome that are being transmitted by the bite of a bloodsucking vector. Often, transmission is the result of insect bites, but mechanical incidents such as vampire bat bites or injury-induced blood contact can also be a source of disease transmission. Human trypanosome infections are in 98% of all cases the consequence of the *Trypanosoma (Trypanozoon) brucei gambiense* parasite that occurs in West- and Central-Africa. The remaining infections are caused by zoonotic *Trypanosoma brucei rhodesiense* parasites. HAT is geographically restricted to sub-Sahara Africa as *T. brucei* parasites strictly depend on completion of their lifecycle inside their final host, i.e., the tsetse (Glossinidae) fly. Occasional HT infections have been reported outside Africa. These cases are classified as non-classic Human Trypanosomosis and are the result of *Trypanosoma (Trypanozoon) evansi* infections. So far, such infections have only been reported in Asia, even though the causative agent is also present in South America, Africa, northern islands of Oceania and even occasionally occurs in Europe. In contrast to HAT, animal trypanosomosis is caused by a wide range of trypanosomes. To start, *T. evansi* is the most widely distributed animal trypanosome. This is a consequence of the fact that the parasite has a very wide transmission vector range combined with a very wide mammalian host range. It has evolved beyond the need to complete its lifecycle inside the tsetse fly and hence has been able to move away from the sub-Saharan tsetse belt. Secondly, *Trypanosoma (Dutonella) vivax* is a livestock parasite that is found in sub-Sahara Africa and South America. Also here, the transmission vector range extends beyond the insect reservoir, as additional mechanical transmission modalities contribute to disease spread. Thirdly, *Trypanosoma (Nannomonas) congolense* is restricted to the African continent. This parasite is mainly transmitted by blood sucking insects from the families of Glossinidae, Tabanidae, Chrysops, Atylotus, and Muscidae. The host tropism for this parasite ranges from domestic animals with economic importance, to wildlife species that harbor parasites as a reservoir, without suffering from severe infection-associated pathology. The latter makes it virtually impossible to envisage the total eradication of this parasite from the African continent. Finally, *Trypanosoma (Trypanozoon) brucei* is considered a vector transmitted parasite with veterinary importance, although the "disease" contribution of this parasite to the overall problem of trypanosomosis is minor. Besides the vector borne trypanosomes, there is also *Trypanosoma (Trypanozoon) equiperdum*, which is a sexually transmitted parasite that causes a deadly disease in equines. Over the years this parasite has occasionally caused havoc in Southern Europe due to illegal or ill-controlled import of infected livestock. The geographic distribution of all HAT and AT causing parasites and general aspects of the immunopathology related to their infections were recently reviewed by Radwanska et al. (1).

In general, host immune responses and immune evasion strategies of salivarian trypanosomes are comparable, although specific host-parasite interactions do govern unique aspect of infection depending upon both parasite and host species. The general hallmarks of infections can be considered to be (i) activation of the innate immune system helping initial parasitemia control, but also driving immune pathology, (ii) antigenic variation of the parasite attempting the evasion of the antibody immune response, and (iii) modulation and destruction of the adaptive immune system (2). In addition, there is the strict regulation of parasite proliferation in the mammalian host that contributes to the intricate control of the host-parasite interaction, as well as the tissue tropism that contributes to the specific characteristics of disease outcome (3). This includes the density-dependent differentiation from the proliferative bloodstream form into the non-proliferating parasite form that is adapted to transmission to the insect vector. In case of HAT, there are two extra components that add to this equation. The first is that during chronic infection, parasites cross the blood brain barrier where they can hide from most of the immune system, causing the deadly neurological pathology outcome of *sleeping sickness*. The second component is the presence of lytic factors in human serum. These offer protection against all trypanosomes except *T. b. gambiense*, *T. b. rhodesiense* and possibly some isolates of *T. evansi*, which have acquired resistance mechanisms that defeat the lytic human serum activity. While some consider these lytic factors to be part of the humane innate immune system, the biochemical trypanolytic activity of human serum factors should probably more adequately be described as a secondary moonlighting effect of the serum protein APOL1, rather than an immune function *sensu stricto*. All these interactions will be further discussed in this review.

With HAT being a zoonotic infection, control of human infection by itself can result in the temporary reduction of victim numbers, but in order to sustain disease control efforts, the animal reservoir component needs to be considered as well. That means that a sub-Saharan landmass of about 10 million km^2, encompassing 36 countries, where trypanosomes

and their transmission vectors are present, should not be considered trypanosomosis-free until the entire livestock herd as well as the wildlife game reservoir is cleared from infections. This is obviously an impossible task to achieve. Hence, for now, disease control relies on detection and treatment of positively diagnosed HAT and AT cases, mass-treatment of economically important animals, and efforts to control insect vectors. Under these conditions, access to a protective anti-trypanosome vaccine would be a huge advantage, and since the 1970's efforts have been undertaken to try and deliver such intervention strategy. Unfortunately, nearly 50 years later, not a single vaccine candidate has made it through a successful field trial. All published efforts undertaken in this context, as well as possible explanations of pitfalls of the applied protocols will be discussed in this review. In short, most vaccine efforts have been focusing on identifying antigens and protocols with a good antibody-induction potential, being driven by the idea that B cell mediated antibody production should be the ultimate weapon against extracellular trypanosomes. However, this strategy does not take into account two issues. The first is that that antibody induction upon antigen stimulation, and B cell memory recall potential during the initiation of infection, are two different immunological concepts. It is the latter mechanism that fails during trypanosomosis. Secondly, trypanosomes have acquired a range of tools to avoid the dangers posed by antibodies as they have adopted to survive in the confined environment of the host blood. This is not a hostile environment, this is where they "live." One way to possibly circumvent these issues is to focus on T cell epitope vaccination rather than B cell epitope targeting (4). Indeed, while the latter is "anticipated" by the parasite and has driven the evolutionary development, triggering T cell memory prior to infection might be a way to boost the immunological "help" in a way that so far has not been part of the immunological pressure that drives the trypanosome genetic drift. T cell vaccination would indeed speed up the initiation of B cell help as well as activate innate inflammatory anti-trypanosome responses upon initial parasite encounter. This idea is based on the fact that trypanosome surface proteins harbor several more conserved hidden epitopes that are not truly immunogenic as such. However, once presented in an MHC-II context by B cells to vaccine induced Th cells, these epitopes could function as the kick-start intermediate link that could tip the balance in favor of the host. Aspects of vaccine failure, vaccine interference by trypanosomes, and ideas dealing with T cell vaccination are being addressed later-on in this review.

TRYPANOSOME GROWTH AND REDUCE PARASITE FITNESS IS CONTROLLED BY INNATE IMMUNE RESPONSES

Sterile immunity against mammalian trypanosomosis never seems to occur, especially not in murine model for salivarian trypanosomosis. The term "resistance" that is often used to describe for example C57BL/6 mice that show a relatively good early parasite load control, and prolonged control of infection, refers only to parasitemia control itself. It does not cover the fact that these mice suffer from severe immunopathology, neither does the term cover the fact that all trypanosome infected C57BL/6 mice will succumb to infection. Similarly, "susceptible" mice such as BALB/c mice, have increased levels of peak parasitemia, often accompanied by a shorter survival time, but exhibit signs of reduced inflammatory pathology as compared to the more resistant strains. It is here that the innate response against infections functions as a double edge sword. While an early IFNγ-mediated inflammatory response will help to control the onset of parasitemia as well as the height of peak parasitemia, it will also be the source of infection-associated anemia, liver inflammation, and the destruction of the adaptive immune response. Hence, it needs to be counterbalanced by a later IL-10 response in order to avoid the occurrence of sever pathology. As the aspects of trypanosomosis-associated immunopathology have recently been reviewed elsewhere (5, 6) this review will rather focus on the immune drivers able to trigger the innate responses that help to control infection. When it comes to the latter, there is a consensus about the fact that IFNγ is the key factor that determines very early-on in infection, whether or not the host will exhibit a relatively good level of parasitemia control (7). This cytokine is first produced by NK cells (8), after which NKT and T cells take over (9). This crucial role for IFNγ was first shown by infecting C57BL/6 mice as well as IFNγ and IL-4 knock-out mice (on the same genetic background) and demonstrating that in the absence of IFNγ, mice became highly susceptible to *T. b. rhodesiense* infection (7). This susceptible phenotype was directly linked to the inability of macrophages to provide a proper anti-VSG response in the absence of IFNγ activation, and their failure to engage in the optimal pro-inflammatory response crucial for the control of early-stage parasitemia (10). This early response to trypanosomosis was shown to be MyD88 dependent (11) and TLR9 dependent (12). Interestingly, exposure of susceptible BALB/c mice to CpG ODN improved their relative resistance, increased their pro-inflammatory cytokine production upon trypanosome exposure and elevated anti-trypanosome B and T cell responses, corroborating the importance of this pathway. At the molecular level, the IFNγ stimulation of the macrophage compartment offers an improved response to the recognition of the trypanosome VSG-GPI anchor (13). The structure of this anchor has been described in detail for both *T. brucei* (14) and *T. congolense* (15) and was show to have a poly-mannose carbohydrate unit that is at the core of the recognition by macrophages. Recognition of this GPI glycosyl core by IFNγ stimulated macrophages results in the induction of TNF, which is the second cytokine that is key to the optimal control of an early peak parasitemia. This was shown in both *T. brucei* (16) and *T. congolense* (17) infections, using TNF knock-out mice as well as treatment with anti-TNF neutralizing antibodies (18). This TNF effect works in conjunction with nitric oxide (NO), which was also demonstrated to be crucial for trypanosomosis control. The role of NO by itself merits special mentioning here as it is also involved in multiple aspects of trypanosomosis-associated immunopathology. First, NO was shown to be the main immunosuppressive macrophage product that suppresses T cell mediate immunity and hence hampers the buildup of T cell memory capacity early on in infection (19). NO is also

able to exert a direct cytostatic activity on *T. brucei* (20) and *T. congolense* (21). For the latter, differences in susceptibility levels between mouse strains was directly linked to their NO response during infection (22). *In vivo* antibody based killing of trypanosomes also required the presence of IFNγ-mediated NO production, most likely affecting parasite fitness during peak parasitemia (23). Interestingly, also TNF has been suggested to negatively affect parasite fitness, showing a direct trypanolytic effect on some trypanosomes. However, for various trypanosome stocks, this effect was not confirmed in the absence of other inflammatory effector molecules such as NO. What does seem to be a more universal observation is the fact that TNF signaling during trypanosomosis is mainly mediated by the TNF receptor-I (TNFp55) (17). As this receptor also drives the induction of immunopathology, this signaling pathway itself can lead to a dual outcome. Interestingly, hosts that respond to acute experimental trypanosomosis by shedding of their soluble TNF receptor-II (TNFp75) suffer much less infection-associated pathology as this mechanism can serve as a two-stage scavenger/release principle. Indeed, during acute TNF production, the presence of the soluble p75 receptor causes the neutralization of the cytokine by forming a receptor-target complex in the circulation. Once the concentration of TNF starts to drop, the complex dissociates and ensures the prolonged presence of the cytokine in the circulation, helping the prolonged control of parasitemia (24).

Most knowledge covering natural and innate resistance to trypanosomosis is derived from experimental mouse infections, which often are initiated by the intra-peritoneal injection of bloodstream form parasites. However, when intradermal infections are initiated with such parasites, *T. congolense* as well as *T. brucei* experiments have shown that the minimum dose required to obtain infection is 100 times higher compared to the dose required for initiation of infection by intraperitoneal injection. This observation suggest that there are improved innate anti-parasite response that govern surveillance at the dermal exposure sites (25). These results could be relevant for vector transmitted infections, as at least in cattle it was shown that it is the innate arm of the immune system, and not the hematopoietic system, that controls parasite growth (26). One obvious cellular player than comes to mind when considering dermal immunology is the presence of neutrophils. Surprisingly, a recent study addressing the role of these innate cells during the onset of infection has shown that they have an infection-enhancing effect, rather than an inhibitory function (27). While the functional role of these cells has not been addressed in other immune sites, their cell number increases dramatically in the spleen of trypanosome infected mice (28).

As for the innate control of human infection, the situation is not totally clear. For a long time, HAT has been called a "lethal" infection, but now the notion is being accepted that there are individuals that carry long-lasting asymptomatic infections. Here, blood parasite levels are so low they are impossible to detect (29). Hence, it could be that a very efficient innate control mechanism manages to subdue infection from the start and does not allow the parasite ever to reach detectable levels in these HAT cases. Till now, it is not clear which mechanisms exactly would drive this resistance as very little HAT-tolerance data is available. While IL-8 has been suggested to be a possible mediator here, TNF and IL-10 have both been associated with disease development (30). An IL-17/Th17 driven susceptibility pathway that has been suggested to occur in *T. congolense* infections (31), has so far not been corroborated to contribute to human HAT related immunopathology or regulation of tolerance.

NON-IMMUNE "INNATE" PRIMATE SERUM FACTORS CAN OFFER BIOCHEMICAL PROTECTION AGAINST TRYPANOSOMOSIS

While the innate immune system of the vertebrate host is a crucial determinant during the onset of a trypanosomes infection, there are additional components that will determine whether or not the initiation of infection is successful. This relates to the fact that the biochemical composition of certain vertebrate host plasma simply does not allow particular parasites to grow. Indeed, while *T. b. gambiense* and *T. b. rhodesiense* are considered severe human pathogens, *T. b. brucei* parasite are unable to survive in human blood due to the particular biochemical composition of human serum. Hence, the latter poses no danger to human health. The fact that the biochemical composition of host serum can determine whether or not a given trypanosome species can successfully initiate an infection, was first proposed when the growth of *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense* were analyzed in the presence of normal (non-immune) human serum (NHS). The fact that *T. b. brucei* is lysed by NHS through an immune-independent mechanism explained why this parasite is unable to infect humans. This trypanolytic biochemical activity is not just found in human serum, but is also present in the blood of other nonhuman primates such as gorilla, baboon, mandrill and sooty mangabey serum, but not chimpanzee, orangutan and macaque (32). In human serum, two different trypanolytic fractions have so far been identified, i.e., TLF1 and TLF2. While both fractions are part of the high-density lipoprotein (HDL) subfraction, they differ in some aspects of their composition, with TLF2 containing a natural IgM fraction (33). What they do have in common is that they contain two compounds that are now generally been agreed upon as necessary for the lytic activity, i.e., Apolipoproteins L1 (APOL1) as the actual lytic compound, and haptoglobin-related protein (Hpr) needed for receptor recognition. While some have called the TLFs as "novel components of the innate immunity" (34), one might argue that they are actually not true immune actors and that the trypanolytic activity of these HDL fractions are biochemical activities that support protection against certain trypanosome species due to a fortunate moonlighting function. Within the context of this review, it is however interesting to highlight the defense principles that TLFs offer against trypanosomes, and how both *T. b. rhodesiense* and *T. b. gambiense* have acquired individual mutations that in turn offers protection against TLFs. First, the primary discovery of how *T. b. rhodesiense* is able to grow in NHS, while *T. b. brucei* is not, started in the 1980s with a long-term genetic analysis project comparing different *T. b. rhodesiense* stocks with various levels of NHS resistance and *T. b. brucei*

stocks. This work culminated in the discovery of SRA (serum resistance antigen), a homolog of the variant surface glycoprotein (VSG) that makes up for the bulk of the trypanosomes surface coat and is crucial for protection against antibody-mediated immune attacks. SRA is constitutively expressed by all *T. b. rhodesiense* parasites when growing in human serum, as "the" molecule conferring resistance to the non-immune lysis by NHS (35). Using *T. b. rhodesiense* SRA as a fishing tool, APOL1 was subsequently isolated from NHS and ultimately proven to be the biochemical compound that carries the actual lytic activity killing activity (36). Today, there is wide consensus over the fundamentals of the APOL1-SRA interaction, the principles that drive APOL1-mediated trypanosome killing in general, and the way *T. b. rhodesiense* neutralizes the killing activity, i.e., by capturing and degrading APOL1 during the process of endocytosis (37). This, in turn, neutralizes the membrane pore forming capacity that APOL1 exerts on intracellular membranes (38). Interestingly, the way that *T. b. gambiense* developed resistance against TLFs is much more complicated and appears to combine multiple mechanisms. First, as *T. b. gambiense* does not have SRA or an SRA homolog, it does not appear to have the capacity to directly neutralize the biochemical lytic activity of the TLFs. In contrast, the resistance mechanism of *T. b. gambiense* involves (i) a reduced binding activity of TLF for its receptor, resulting in a reduced TLF uptake, (ii) increased cysteine protease activity involved in APOL1 breakdown, and (iii) a role for TgsGP, a VSG-like *T. b. gambiense* specific glycoprotein (39). The latter was elegantly proven by generating a *T. b. gambiense* TgsGP knock-out parasite line as well as a rescue mutant, and showing that survival in human serum directly correlated to the presence of the targeted gene (40). Interestingly, while these data show that *T. b. gambiense* went through several evolutionary steps to become an omnipotent infective *T. brucei* parasite, some primates have undergone APOL1 evolution so that it confers resistance against *T. b. gambiense*. This is the case for the West African Guinea baboon that lives in a *T. b. gambiense* endemic area, and has a gene encoding for an APOL1 variant that confers protection against both *T. b. brucei* and *T. b gambiense* (41). These examples are a clear indication of how the arms race between parasites and host innate defense system can shape the genome of both partners, and how even non-immune "innate" responses can be a key factor in disease resistance or susceptibility.

TRYPANOSOMES CONTROL THEIR POPULATION DENSITY BY QUORUM SENSING

The innate host immune responses obviously play a crucial role in the control of the initial onset of trypanosome infections. However, it is known that the peak blood parasite load in experimental infections is not just linked to host immune factor activity, but also depends on intrinsic characteristics of the parasite. Indeed, early experiments using nu/nu T cell knock-out mice, and later experiments in μMT B cell deficient mice have indicated that maximum parasite density during peak parasitemia is not significantly affected by either of these cell populations and appears to be regulated by a quorum sensing mechanism of the parasite itself. In *T. brucei*, this mechanism seems to drive the differentiation from dividing slender form parasites into non-dividing stumpy form parasites, hence halting the proliferation of the population (42). By showing that the system was operational *in vitro*, it was concluded at the time that this sensing mechanism was independent of host factors, and was dependent on the secretion by the parasite of an elusive stumpy inducing factor (SIF) (43). SIF was shown to trigger cell cycle arrest in the G_1/G_0 phase preceding cell differentiation, potentially involving a cAMP signaling pathway. Without proper identification of the exact nature of SIF, the story of quorum sensing in trypanosomes remained silent for about 15 year, until the recent use of a genome-wide RNAi library screen identified the signaling components that drive stumpy formation. Candidate genes were subsequently validated, confirming their role in density sensing *in vivo*, leading to the identification of the putative RNA-binding protein RBP7 as a key component in both quorum sensing and cell-cycle arrest (44, 45). Interestingly, the quorum sensing observed in *T. brucei* is also observed in *T. congolense* although in the latter, density-dependent cell-cycle arrest does not result in actual stumpy form formation and does not follow exactly the same gene regulation profile (46). It does however prepare the parasite for transmission to its definite insect host. Under field conditions, *T. brucei* and *T. congolense* coinfections are not uncommon, raising the question how the quorum sensing factors of one parasite would affect the other. The results of combined experiments showed the systems are indeed similar as the *T. congolense* genes can complement the pathway in *T. brucei*. In addition, conditioned culture medium from *T. congolense* promotes stumpy formation of *T. brucei in vitro*, and *T. congolense* co-infection accelerates differentiation to stumpy forms in *T. brucei* (47).

Most recently, the investigation of the quorum sensing mechanism in *T. brucei* has delivered an exciting breakthrough than can explain a number of results that previously had been difficult to align with the view that SIF is purely dependent on the parasite, without any involvement of the host. Indeed, while trypanosomes do not have G protein-coupled receptors the Matthews laboratory identified a GPR89-family protein that regulates stumpy formation in *T. brucei* (48). *Tb*GPR89 is expressed on the surface of proliferating slender form parasites and is able to sense the presence of SIF, which so far was only identified as a small <500 Da non-proteinaceous heat-stable factor. Based on its structure, *Tb*GPR89 was predicted to be an oligopeptide transporter and given that the secretome of *T. brucei* contains a number of oligopeptidases, the most straight-forward explanation for the density-dependent stumpy transformation is the fact that as trypanosomes accumulate in their environment (*in vitro* or *in vivo*), so does the oligopeptidase activity. This would subsequently create a pool of peptide breakdown products from surrounding proteins of which specific oligoptide compounds can be detected by the parasite. As this signal increases, the parasite slows down its own growth, avoiding the killing of its host by hyper parasitemia and at the same time preparing itself for transmission to its insect vector (49).

There are still arguments that would defend the existence of a second density independent stumpy development pathway in *T. brucei* (50), in which expression site regulation is the key to the transformation process, but only the GPR89 driven system would solve the question that has been raised by several immunologists when looking at the quorum sensing mechanism of trypanosomes, i.e., how is it possible that when comparing parasitemia of a cloned parasite in different mice with different genetic backgrounds, the peak parasitemia level is defined by the genetic background of the host and not the parasite? Indeed, when comparing the growth of identical pleomorphic *T. b. brucei* AnTat 1.1E parasites in a range of different mouse strains (including knock-out strains), peak parasitemia density directly correlates to the intrinsic inflammatory bias of the host. Mice with a natural low inflammatory induction potential such as BALB/c or C3H/HeN mice show a much higher peak parasitemia density than mice with high inflammatory induction capacity such as CBA/Ca or C57BL/6 mice (24). Also, when B cell deficient mice on a C57BL/6 or BALB/c background are challenged with identical *T. brucei* parasites, the peak parasitemia density is very different depending on the genetic background of the host, but not affected by the presence or absence of antibodies (51), once again showing that the stumpy differentiation and the control of peak parasitemia density is affected in the first place by differences in host genetics. The answer could be that extracellular oligopeptidases from inflammatory immune cells and inflammation damaged tissue contributes to the formation of the peptides that make up the SIF-pool. From an evolutionary point of view that would mean that trypanosomes have "learned" not just to sense their own density, but at the same time can sense the inflammatory state of the host. When either hyper-parasitemia, or hyper-inflammation risks killing the host, the parasite population goes into growth arrest and counts on vector transmission for ultimate survival.

TRYPANOSOMES AVOID ANTIBODY-MEDIATED KILLING BY DYNAMIC SURFACE COAT INTERACTIONS

Once trypanosomes have established a successful infection, it is obvious that due to the extracellular nature of these infections, antibodies are going to be the main immune component that the parasite will have to deal with. It is generally accepted that the pressure exerted by antibodies has resulted in the fact that extracellular trypanosomes have developed a very sophisticated mechanism of antigenic variation, which allows the switching of the antigenic type of their glycoprotein coat at regular time intervals. The mechanisms that are being used by trypanosomes to do this are remarkably similar to the mechanism that the host uses to generate antibody diversification (52). Indeed, while both trypanosome "cells" and B cells use a system of mono-allelic expression to ensure that only one VSG (trypanosomes) or one B cell receptor (BCR) is expressed by a single cell at any given time, both the entire trypanosome population within the host, as well as the entire hosts' B cell population, both exist out of multiple different clones, representing an overall heterogenous population. In addition, proliferation in combination with (somatic) hyper-mutation allows both VSGs and BCRs to play a game of hide-and-seek, ensuring chronic infections and resulting in the continued undulating parasitemia occurrence that is typical for natural trypanosome infection. The occurrence of antigenic variation in human trypanosome infections and the implications for sero-diagnostics has been covered by others (53), and both the molecular mechanisms (54) as well as the *in vivo* dynamics of antigenic variation of the trypanosome surface coat have been described and reviewed in detail in the past (55), and hence are not the target of this review. Recently, the diversity of VSG genes in *T. brucei* was addressed during chronic infections, using the application of long read sequencing (56). Obtained data have confirmed the existence of a VSG repertoire that cannot be exhausted by immune pressure, due to the continuous generation of new mosaic genes that had been reported earlier (57). In addition, this recent data confirms that every parasitemia peak consists of multiple trypanosome populations expressing multiple VSGs, that sometimes are closely related. Finally, this data also demonstrates that there is significant expressed diversity that follows a semi-reproducible pattern over time, when infections in different mice are compared (56). Hence, in the context of this review it is interesting to recapitulate how the antibody arm of the mammalian immune system interacts with the trypanosome surface, and how the trypanosome manages to evade immune destruction by the antibody response. First, it is important to stress that for most trypanosome species, the VSG coat consist out of a densely packed layer of surface glycoproteins, stacking an estimated 10^7 identical copies of homodimer molecules evenly distributed over the parasite surface. Hence, this coat has long been considered as an "impenetrable" barrier. More recent structural data have shown however that the real barrier function that would protect the plasma membrane is only being provided by the glycosylation present at the base of the VSG, and that multiple invariant surface proteins are actually fully accessible to the host antibody response. These new data highlight the gap in our understanding of how the VSG coat really works (58). The gap in understanding is further highlighted by the observation that trypanosomes that express two different VSG variants at the same time, an event that could take place at the time point where one VSG coat is being replaced by a new coat, fail to trigger an efficient B cell response (59). Hence, while expressing a mosaic coat could be a good way to evade antibody-mediated elimination, this is not what trypanosomes have evolutionary developed as escape strategy. Interestingly, a VSG mosaic coat does not deliver intrinsic constraints that would hinder BCR crosslinking and subsequent B cell activation. Indeed, it has been estimated that the VSG density of intact *T. brucei* parasites is 20 times higher than the density required for BCR cross-linking. So, even if a VSG mosaic coat consists out of two antigenically district variants, each of the variants should in theory be able to mediate regular B cell activation, which is apparently not what happens (60). Yet, experimental infections in distinct mouse strains, as well as their F1 offspring, have shown that susceptibility to infection in terms of limited parasitemia control and shortened survival can be linked to the impaired capacity to mount a proper anti-VSG

response. Interestingly, F1 generations showed that the capacity to mount a good anti-VSG antibody response is inherited as a dominant trait, while survival time remained similar to the susceptible parental strain (61). These data showed that antibody-mediated parasitemia control and disease susceptibility in terms of survival are controlled by different mechanisms. Furthermore, these finding were corroborated by studying *T. b. rhodesiense* infections in mouse chimera models, indicating a role for IgM in parasitemia peak control but not survival (62). With the arrival of mouse knock-out technology, the role of antibodies in trypanosomosis control was reassessed in various models. First, it was shown that in experimental *T. b. brucei* infections, IgMs do play a role in peak parasitemia clearance (but not the control of the peak height itself) in the relatively virulent AnTat 1.1E pleomorphic needle transmission model. In contrast no obvious role was observed in the control of the low virulent TSW196 field isolate, or during tsetse fly initiated AnTat infections (51). Interestingly, B cell deficient μMT mice (either on a susceptible BALB/c background or a more resistance C57BL/6 background) failed to clear peak parasitemia levels. Hence, one can conclude that while IgMs do help to control more virulent infections, there are other antibodies that can take over the role in parasite clearance when IgMs are absent. In a model for *T. b. rhodesiense*, it was shown that IgM antibodies have a better trypanosome agglutination potential, but that the IgG fraction provided a better protective response in serum transfer experiments (63). This could be related to the better tissue penetrating capacity of the latter, which could be important during natural and chronic HAT infections. Next, the role of IgMs was addressed in experimental *T. congolense* infections using the chronic Tc13 model. Here it was found that while C57BL/6 μMT mice were completely susceptible and succumbed to infection following an uncontrolled first peak of parasitemia, IgM deficient mice survived on average nearly 4 months, similarly to the fully immune competent controls. Interestingly, as already outlined in the section above on innate anti-trypanosome responses, infection control in this model was shown to be dependent on the presence of an INFγ/NO/TNF inflammatory environment. Most likely, this environment reduces parasite fitness, allowing antibody-mediated clearance without the strict requirement for the participation of IgM antibodies (23). It could be argued however that as the *T. b. brucei* setting, intrinsic parasite virulence is also a factor here and that in case of a more virulent *T. congolense* infection, there could be a supporting role for IgMs in parasite clearance. This has not been tested so far. For experimental *T. evansi* infections the results appear to be the somewhat opposite of what was reported for *T. congolense*. Here, both C57BL/6 μMT mice and IgM deficient mice showed a very high level of susceptibility, being unable to control the first peak of parasitemia. Hence, in these infections IgGs are not able to take over the protective role of IgM (64). Whether or not these results were affected by the intrinsic virulence level of the stabilates used, remains to be tested. Finally, in case of *T. vivax*, infections were done in μMT mice as well as a number of cytokine deficient mice, but not in IgM deficient mice. Results available so far show that the combination of anti-trypanosome antibodies and an inflammatory immune environment, in particular the presence of TNF, is needed for proper parasitemia control, very similarly to the results described for *T. congolense* (65).

So far, IgMs appear to play a crucial role in all trypanosome models at least in the control of more acute infections. Hence, it is interesting to speculate on the exact functional mechanisms involved here, which could involve a role for the Fcα/μ receptor. This Fc receptor has been shown to be important for the endocytosis of IgM opsonized microbes (66), although it has not been studied in the case of trypanosomosis. With no evidence for a role of complement-mediated lysis of trypanosomes in *in vivo* infection control (this is discussed in detail in the section below), it appears indeed that phagocytosis, in particular by Kupffer cells, of opsonized parasites is the only way that antibodies can help in the clearance of peak parasitemia numbers (67). Additional data suggest that the presence of an inflammatory environment aids this process, most likely due to the fitness reducing effects of TNF and NO, either by themselves or when combined. Interestingly, by artificially blocking VSG synthesis using an RNAi approach, it was shown that the process of macrophage phagocytosis was much more efficient (68). This finding suggests that VSG recycling is a key process in the defense of the parasite against the attack by the mammalian antibody response. This hypothesis is supported by an observation dating back to 1979, when D. J. Barry first described that upon exposure of trypanosomes to homologous antiserum, VSGs moves to the flagellar pocket in a temperature dependent process (69). The presence of a phospholipase C enzyme that could release soluble VSG by cleaving its GPI anchor in the flagellar pocket, was later suggested also to be part of the VSG-membrane recycling system (70). The detailed recycling pathway of VSG was subsequently described by two independent groups (71, 72), showing that trypanosome have an extremely fast rate of endocytosis resulting in the turnover of their VSG coat within 12 min and the formation of 6–7 clathrin-coated vesicles per second, that bud from the flagellar pocket and deliver the membrane content to RAB5-positive early endosomes. Next, VSG is recycled through RAB11-positive recycling endosomes that return the VSG to the surface. It is now believed that trypanosomes use this system not only for the uptake of nutrients contained in the fluid phase of the vesicles and bound to exposed membrane receptors, but that in addition it allows the parasite to "clean" the coat from bound antibodies. This protects the parasites from damaging antibody functionality as long as the cells are in an optimal metabolic state. This hypothesis was very elegantly shown by Engstler et al. when they reported that Ig-VSG membrane immune complexes are passively sorted to the posterior cell pole, where they are endocytosed through the flagellar pocket. This process requires forward cell motility and results from the hydrodynamic forces that are encountered due to the directional "swimming" motion of the trypanosomes (73). It was shown that *in vitro* this molecular flow movement protected trypanosomes from complement-mediated lysis. The same principle would also protect the trypanosome from efficient antibody-mediated phagocytosis *in vivo* as surface-bound antibodies would function as molecular sails that would direct immune compromised VSGs toward the flagellar pocket for "cleaning" or replacement (74). It is obvious that as the immune system would mount an increasing

antibody response with time, this system would become saturated and eventually fail. However, this delay in antibody-mediated destruction possibly offers an extended time window during which antigenic variant can take place. This will increase the chance that a surface VSG appears that is distinct enough to avoid recognition of the parasite by existing circulating antibodies, allowing the survival of some parasites within the population.

TRYPANOSOMES AVOID ANTIBODY-MEDIATED KILLING BY DESTRUCTION OF THE HOST B CELL COMPARTMENT

Despite the importance of antibodies in the control of extracellular trypanosome parasites, there is relatively little data available describing the actual role and immunobiology of B cells in either experimental mouse trypanosomosis or natural human/cattle infections. In cattle, B cell responses have been compared between trypanotolerant N'Dama and susceptible Boran cattle, in order to try and link humoral immune response to parasitemia control. While it has already been mentioned that in cattle actual parasitemia control was found to be in large regulated by the innate immune response (26), these comparative studies did show that trypanotolerant cattle were able to produce higher anti-VSG IgG serum responses (75). In addition, these animals had more circulating lymphocytes that could be activated *in vitro* to undergo differentiation into IgM- and IgG secreting cells. In contrast, trypano- susceptible cattle showed higher frequencies of spleen IgM secreting cells producing antibodies that were not directed toward the trypanosome VSG coat. Interestingly, in both *T. congolense* and *T. vivax* cattle infection, $CD5^+$ B1 cells were found to be the main source of poly-reactive IgM responses, producing antibodies that bind to β-galactosidase, ovalbumin and ferritin (76). This polyclonal B cell activation is considered to be part of the "immunosuppression" that characterizes trypanosome infections, as it *de facto* reduces the chance for an efficient specific immune response to be developed against crucial parasite antigens. Polyclonal B cell activation has also been reported in experimental infections with *T. b. brucei* and *T. b. rhodesiense* in mice (77). While the latter report linked this immunological phenomenon to the presence of the VSG, the exact mechanism driving the activation has so far not been unraveled. Besides polyclonal activation, there are two other B cell biology observations that merit attention in the context this review, i.e., the non-classic regulation of Ig class switching, and the destruction of the B cells compartment that constitutes the main pathology problem for the immune system during chronic infections. Unusual Ig class switching was first described in a study that measured anti-VSG isotype profiles during *T. b. rhodesiense* infections in mice. Here is was found that both fully immune competent C57BL/6 mice as well as T cell deficient nu/nu mice were able to mount a very similar switched anti-VSG response that mainly consisted out of IgG1, IgG2a, and IgG3 antibodies. This occurred in the presence of IFNγ and IL-2, but the absence of IL-4 and IL-5. However, when repeating these measurements in IL-4 knock-out mice, a significant decrease in IgG1 titers was observed, indicating a role for this cytokine in the regulation of B cell activity, independent of antigen specific Th1 or Th2 cells (78). In the same context, IgG switching was also observed in experimental *T brucei* infections in both WT and nu/nu BALB/c mice, with IgG1, IgG2a, IgG2b, and IgG3 isotype antibodies being produced independent of conventional T cell help (79). Interestingly, in both models the lack of T cell help did not seem to hamper parasitemia control, while the lack of Il-4 and the subsequent capacity to drive an IgG1 B cell differentiation pathway also did not affect parasitemia control. One example where B cell regulation was shown to be a crucial arm of parasitemia control was in chronic *T. congolense* infections in mice. Here Bam32, responsible for downstream signaling linked to BCR crosslinking, was shown to be crucial for prolonged parasitemia control, albeit redundant for early peak parasitemia elimination (80). This observation aligns with the finding that *T. congolense* infections cause a sustained disruption of the B cell homeostasis in bone marrow and spleen, and that the virulence index of different stocks in experimental mouse models correlates with the potential to drive B cell destruction (81). When it comes to immune destruction of the B cell compartment, this has also been documented in detail for *T. vivax* (82) and in more detail for *T. brucei* (83). In the latter model it has been shown that the early loss of B cells is linked to an excessive inflammatory IFNγ response (84), can involve the NK cell mediated killing activity (85), is observed both at the level of the bone marrow and spleen (86) and takes several weeks to be restored after drug treatment of experimentally infected mice (87). Also here, infection-induced B cell destruction seems to be associated to parasite virulence as mice that are infected with very chronic *T. b. gambiense* parasites do not seem to suffer the same B cell depletion problem as those infected with a much more acute *T. b. brucei* strain (88).

SERUM COMPLEMENT FACTORS MEDIATE PARASITEMIA CONTROL *IN VIVO*, BUT NOT TRYPANOLYSIS

Immunoglobulins only have a limited range of function they can perform by themselves. In essence, good antibodies are proteins that bind their target with high affinity and specificity. For trypanosomosis control, both antibody-mediated phagocytosis (discussed above) and antibody-mediated complement lysis are quoted throughout the literature. However, four decades of complement research in the context of trypanosomosis have shown that "lysis" is not the mechanism that mediates parasitemia control during *in vivo* infection. The reason for the persistence of this misconception about complement lysis of trypanosomes has probably to be attributed to the fact that *in vitro* antibody-mediated trypanolysis assays are being used as diagnostic tools for the detection of active infections. Indeed, when cultured or purified trypanosomes are incubated *in vitro* with high amounts of complement-rich guinea pig serum and plasma from infected individuals or animals, trypanosomes tend to lyse over time proportionate to the amount of anti-trypanosome antibodies present in the donor serum (89). This phenomenon has been very clearly described in the 1970's in

the correct context of the particular *in vitro* conditions for *T. congolense* and *T. brucei*, and the authors also immediately discussed the limitations of the observation with respect to the *in vivo* situation (90). Also the involvement of the alternative activation of the complete cascade under *in vitro* conditions with the role of C3 and the formation of covalently immune complexes between released soluble sVSG and C3b has been described, without any claim that this would represent the system that controls parasitemia *in vivo* (91). In fact, over the years, a wealth of data has been published that shows that parasitemia control *in vivo* occurs totally independent from the lytic complement cascade, and that trypanosomes have a number of tools at their disposition to efficiently prevent complement-mediated lysis of actively dividing bloodstream form parasites. Maybe the most compelling data in this context comes from experimental infections in animals that simply do not have the capacity to activate the full complement cascade due to genetic mutations. For example, AKR mice as well as B10.D2/oSnJ mice are both deficient for C5. Hence, while the complement cascade can be initiated in these mice with the formation of the opsonin C3b molecule, the cascade cannot progress toward the formation of the membrane pore forming 10–16 C9 complex. Therefore, the fact that B10.D2/oSnJ mice are able to control successive waves of *T. b. rhodesiense* infection proves that complement-mediated lysis is not crucial in the process of either peak parasitemia control, or peak parasitemia clearance (92). Similarly, these C5-deficient mice were capable of controlling *T. musculi* infections (93), as well as *T. congolense* infections (94). Also AKR mice were used to study the progression of infection in models for both *T. b. rhodesiense* (95) and *T. vivax* (65), showing again that peak parasitemia control as well as peak parasitemia clearance occurs in the absence of C5, and by consequence in the absence of complement-mediated trypanolysis. The lack of C5 involvement in trypanosomosis control is further corroborated by the fact that *T. congolense* infections hardly affected plasma C5 levels in a range of different mouse models, including highly susceptible and highly tolerant strains (96). Interestingly, the latter study did report that trypanosome infections resulted in a rapid decline of C3 in all strains tested. This confirmed earlier results that had shown that while C8 levels are unaffected during infection C1, C1q, and C3 levels all decrease in plasma of *T. congolense* infected cattle (97) as well as in *T. vivax* infected cattle (98). Finally, it is important that these experimental mouse and cattle data also reflect the situation in humans. Indeed, while *T. b. gambiense* does activate C3 and binds C3b on its surface when incubated with human serum, it does not result in the formation of C5 or the polymerization of C9. Hence, trypanosomes are not lysed as the complement cascade does not continue beyond the establishment of C3 convertase (99).

So, could there still be a role for complement in the control of trypanosomosis? The "yes" answer relates to the fact that besides lysis, the initial stages of complement are crucial for the generation of the C3b component, a powerful opsonin that binds covalently to its target (100). By interaction with plasma Factor H and I, C3b can be converted into iC3bi (or C3bi) which is a target for the CR3 (CD11b/CD18) receptor involved in pathogen phagocytosis (101). In case of *T. congolense* infections it has been described that mice with effective prolonged parasitemia control and low parasitemia peaks (C57BL/6) have a faster and greater C3b production than mice that intrinsically fail to control the infection with the first 2 weeks of a parasite challenge (BALB/c) (102). This activity correlates with the finding that the CR3 receptor is a major player in the endocytosis of *T. congolense* parasites after opsonization of the parasite by IgM antibodies (103). Interestingly in an innate immune context is the finding that CR3-mediated phagocytosis is also directly linked to macrophage TNF production, an inflammatory cytokine that as described above has a profound negative impact on trypanosome fitness. Complement associated phagocytosis has also been described for *T. brucei* (104), and more recently the use of C3 knock-out mice confirmed a role for complement in peak parasitemia clearance during *T. brucei* infections (85). All these data confirm older results showing (i) that early stage complement cascade activation is important for parasite clearance (105), (ii) that hepatic phagocytosis of trypanosomes depends on the presence of C3, and (iii) that *in vivo*, no intravascular lysis of parasites is observed during events of immune clearance (106). However, simply to illustrate the complexity of the immune system, it should be said that in the presence of IgM opsonization of *T. brucei* parasites, treatment of mice with CVF (cobra venom factor) that neutralizes C3, did not ablate the capacity of macrophages to phagocytose trypanosomes. This shows that in immune competent mammals, multiple mechanism are in place to deal with peak parasitemia clearance (107).

Taken that C3b/iC3b deposition on the VSG surface coat can result in hepatic phagocytosis, the trypanosome must have defense mechanisms in place in order to ensure that this system does not lead to population elimination. As already outlined above, rapid VSG circulation comes into play here, as a defense system against any host compound binding to the surface (108) and as a tool for complement surface clearance in particular (109). Secondly, the densely packed nature of the VSG coat itself offers physical protection against any possible complement-mediated lysis (110). Finally, it has been proposed that trypanosomes have two more defense systems in place that could help the fight against host complement. First, trypanosomes were shown to express a homolog of the *Leishmania* GP63 surface protease that has been shown to confer resistance to complement-mediated lysis (111). Secondly, *T. brucei* was shown to bind human complement regulatory protein C4BP. Acquisition of this factor on the surface of pathogens is correlated to the downregulation of complement activation (112). Hence, together these mechanisms provide the trypanosome with adequate tools to confer resistance to complement mediate lysis, but at the same time allow the immune system to clear parasites trough phagocytosis in the liver. The latter would be particularly helpful when parasite fitness is reduced due to ongoing inflammatory responses triggered by the innate immune system, and during events of parasite growth inhibition that result from the quorum sensing mechanisms described above.

T CELLS CONTRIBUTE TO THE MAINTENANCE OF AN ANTI-TRYPANOSOME INNATE INFLAMMATORY ENVIRONMENT

Since the very beginning of trypanosome immunology research, T cells have been considered crucial for proper trypanosomosis control. How and why this is the case, strongly depends on the model studied and the definition of infection control. For example, when calves were depleted from $CD4^+$ T cells and trypanosome control was studied, the effects observed were strongly dependent on the intrinsic trypanotolerance of the breed. While T cell depletion always had a very severe negative effect on antibody production, this did not impact parasitemia control in trypanotolerant N'Dama cattle. In contrast, the same treatment had a profound negative impact on parasitemia control in susceptible Boran cattle (26). This finding suggests that while T cell mediated responses can help to bring parasitemia under control in certain conditions, there are T cell independent factors that can be responsible for intrinsic trypanotolerance. $CD8^+$ T cell depletion in susceptible Boran cattle did not affect parasitemia control (113). N'Dama/Boran chimera experiments in cows confirmed that intrinsic parasitemia control was controlled by a hematopoietic independent mechanism, while disease control, i.e., anemia development, was in contrast dependent upon the hematopoietic system.

In mice, control of trypanosome infectious does not exactly mirror the observations of cattle, but there are parallels. A first interesting observation is the T cell deficient nu/nu mice on either BALB/c or C57BL/6 background have no problems in controlling parasitemia in comparison to fully immune competent mice (51, 79). In fact, when it comes to pathology control, C57BL/6 mice that lack T cell altogether, or just $CD8^+$ T cells, suffer less from infection-associated anemia (9), and have a prolonged lifespan in case of nu/nu mice when infected with *T. b brucei* or *T. b. rhodesiense* (9, 114). This dichotomy between parasitemia control and pathology/survival most likely reflects the complex role T cells play in (i) sustaining the innate inflammatory environment needed for proper parasitemia control, (ii) the immunopathology associated with prolonged inflammation, and (iii) in case of $CD4^+$ T cells, their role in helping a T cell dependent anti-trypanosome antibody response. As also discussed above, antibodies involved in trypanosomosis control in mice comprise both a T cell independent and T cell dependent fraction. Indeed, while even nu/nu mice are able to raise VSG specific antibody responses, fully immune competent mice often—but not always—show a better response (115). Interestingly, in fully immune competent mice T cell responses to trypanosome VSG are not limited to the hypervariable regions but also cover the less variable regions of the N-terminal domain that are inaccessible for antibodies. At the same time however, it appears that during experimental infections no T cell are raised against epitopes located in the relatively invariant C-terminal part of the VSG (116). This opens an interesting hypothesis for future vaccine attempts which is also discussed further in this paper: could it be that trypanosomes evolved to have an immune evasion mechanism that prevents exposing conserved epitopes in an MHCII context, so that during infection no T cell memory is developed against such structurally important domains? While these domains are constrained with respect to sequence variation due to their functional importance for VSG structure, they are buried at the base of the VSG and hence are relatively safe from antibody recognition. If, however such epitopes were to successfully recognize by the T cell immune compartment after antigen processing, they could serve according to the hapten-carrier concept as $CD4^+$ T helper cell generators that would provide help to all anti-VSG B cells, against all surface exposed epitopes. This would negatively affect the fitness of the trypanosome. Hence, while such negative selective pressure would favor the acquisition of non-immunogenic conserved regions, the artificial induction of T cell responses against these epitopes prior to infection might tip the balance in favor of the host, in a way that trypanosomes have not been evolutionary prepared for (4).

Beside the possible help to B cells, it is clear that T cells provide crucial help to the maintenance of a correct cytokine environment, needed to help control parasitemia through innate related mechanisms. Indeed, it has been shown that in C57BL/6 mice that exhibit a relatively good parasitemia control, infection control is associated with the induction of Th1 $CD4^+$ responses, characterized by the production of IFNγ and IL-2 and the absence of IL-4 (117). The importance of this response for macrophage activation and parasitemia control was confirmed by the fact that C57Bl/6 IFNγ knock-out mice are highly susceptible for trypanosome infections, as already indicated in the section that covered the innate protection against the parasite. In contrast, IL-4 knock-out mice showed a phenotype that was identical to fully immune competent mice (7). This drive toward Th1 differentiation has been correlated directly to the splenic DC antigen presentation of VSG that coincides with IL-12 secretion (118). At first, these results might seem conflicting to the results observed in trypanosome infected nu/nu mice, where the latter are characterized by a normal parasitemia control. It is however important to emphasize the fact that infection-induced IFNγ production does not solely result from the presence of activated CD4+ T helper cells. In particular during early infection, it was indeed shown that the first cells to respond with a significant IFNγ secretion are NK cells, subsequently followed by NKT cells and only later by Th1 $CD4^+$ T cells and $CD8^+$ T cells. Hence, it is possible that as far as the early need for IFNγ is concerned, nu/nu mice have a compensatory mechanism in which the NK/NKT compartment delivers the correct cytokine environment to control early parasitemia by a combination of inflammatory cytokines and T cell independent antibodies (9). Finally, one line of thinking that also could explain in part the results observed in overall comparative mouse infection, is that so called "wild type" mice undergo an immune suppression of T cells that makes them *de facto* closely resemble functional T cell deficient mice. In this case, trypanosome infected WT mice would indeed functionally resemble nu/nu mice in some respects, despite the presence of T cells in the lymphoid circulation.

TABLE 1 | Summary of vaccine candidates reported in literature.

Type of vaccine	Antigen	Antigen preparation	Boosts/host	Time gap boost-challenge	Parasite load	Outcome	References
Intra-muscular	*T. b. rhodesiense* FP	Parasite isolated	3/cattle	14 days or more	Natural exposure	Partial protection	(120)
I.p.	*T. b. brucei* AnTaR FP	Parasite isolated	3/mouse (Balb/c)	3 weeks	500–10^3	Partial/no protection	(121)
I.p.	*T. b. brucei* MITat ISG65, ISG75	Recombinant protein	3/mouse (C57bl/6)	11 days	10^4	No protection	(122)
I.p.	*T. b. brucei* ISG75	Plasmid DNA	1/mouse (Balb/c)	175 days	500	Partial protection	(123)
I.p.	*T. b. brucei* GuTat10.0 Ca^{2+} ATPase TBCA2	Recombinant protein	3/mouse (Balb/c)	6 weeks	10^6	No protection	(124)
Sub-cutaneous	*T. brucei* (UTRO010291B) Tubulin rich fraction	Parasite isolated	3/mouse (no strain indication)	Not indicated	10^3-10^5	Partial (cross-species) protection	(125)
Sub-cutaneous	*T. evansi* (STIB806) b-Tubulin	Recombinant protein	3/mouse (Balb/c)	6 days	10^3	Partial (cross-species) protection	(126)
Intra-muscular	*T. evansi* (EU483116) β-Tubulin	Plasmid DNA	2/mouse (Swiss albino)	35 days	10^3	No protection	(127)
Sub-cutaneous	*T. evansi* (EU483116) β-Tubulin	Recombinant protein	2/mouse (Swiss albino)	14 days	10^3	No protection	(128)
Sub-cutaneous	*T. evansi* (STIB806) Actin	Recombinant protein	3/mouse (Balb/c)	6 days	10^3	Partial (cross-species) protection	(129)
Sub-cutaneous	*T. congolense* CP1 & CP2	Recombinant protein	4/cattle (Boran)	1 month	Tsetse bite	Improved recovery	(130)
Intra-muscular	*T. b. brucei* Sialidase	Plasmid DNA	1/mouse (Balb/c)	175 days	500	Partial protection	(131)
I.p.	*T. congolense* Sialidase	Recombinant protein	4/mouse (Balb/c)	10–14 days	10^4	Partial protection	(132)
I.p.	*T. b. brucei* AnTat GPI	GPI-Liposomes	2/mice (C57bl/6 and KOs)	3 weeks	5×10^3	Cross-species anti-pathology	(133)

I.p., intraperitoneal; FP, Flagellar Pocket; ISG, Invariant Surface Glycoprotein; GPI, glycosylphosphatidylinositol; CP, Cystein proease.

EXPERIMENTAL ANTI-TRYPANOSOME VACCINES HAVE SO FAR NOT BEEN TRANSLATED INTO APPLICABLE VACCINES

In 2011, we published a comprehensive overview of anti-trypanosome vaccine data that was available at the time (119). We concluded that while there is a fair amount of "promising" results in the literature, there is little prospect of seeing a successful field applicable vaccine any time soon. Ten years later, a number of additional results have been published, but it appears that the latter statement still stands. An overview of the currently reported anti-trypanosome vaccine data is provided in **Table 1**.

The rationale behind most anti-trypanosome vaccine efforts is to vaccinate a host with invariant trypanosome proteins or a mix of such targets. Given the role of the flagellar pocket in nutrient uptake, and the presence of a number of surface receptors and invariant surface molecules such as the LDL receptor and the HpHb receptor, Invariant Surface Glycoproteins ISG65 and ISG75, and SRA (134), the FP was targeted in two independent approaches. In one report, cattle were inoculated with crude antigen extracts derived from the FP of *T. b. rhodesiense*, and subsequently exposed through a possible natural challenge with *T. congolense* or *T. vivax* by releasing the animals in their natural field habitat (120). This resulted in a significant drop in infection prevalence at the end of the observation period that lasted 15 months. In a more controlled mouse vaccine study, FP vaccination was conducted using highly purified material from *T. b. brucei* parasites. Borderline protection was observed when mice were challenged with 500 live bloodstream form parasites, with 60% of mice not developing parasitemia for a period of up to 100 days (121). No protection was observed however when mice were challenged with 1,000 parasites or more. Important is that the ultimate idea behind a vaccine is to deliver memory recall responses for prolonged periods of time. In the setting used here, this was not tested as the waiting period between the last boost and the parasite challenge was only 21 days. Hence, immune modulation events, including innate responses triggered by repeated adjuvant exposure, could have contributed to the limited protection observed here in a low-dose challenge setting.

Upon discovery of the invariant trypanosome surface proteins ISG65 and ISG75, they were considered as viable vaccine candidates. While ISG75 was shown to be a good antibody inducer, no protection was observed in a vaccine setting using a homologous parasite challenge, despite the very short 11-day waiting period between the last boost and the actual parasite

injection (122). Next, a DNA vaccine approach targeting ISG75 was tested. Here, a single vaccination was followed 175 days later by exposure to a low-dose challenge with 500 *T. b brucei* GVR35 parasites. The results obtained were virtually identical to the mouse FP vaccine low-dose challenge experiment and showed 60% protection against parasitemia for a period of at least 60 days (123). As in the case of the FP vaccination, this outcome was associated with a strong IgG2a antibody presence, indicative of an IFNγ driven cytokine environment. These results do not conclusively point to the involvement of an antibody effector mechanism and could equally well-indicate that biasing the host toward a strong inflammatory mediated immune regulatory environment, favors protection against low-dose parasite exposure, due to a more efficient help to the innate anti-trypanosome immune response. A very similar conclusion can be made for an anti-trypanosome vaccination that used the Ca^{2+} ATPase TBCA2 antigen as a target in BALB/c mice. This trypanosome membrane pump was formulated in a *Vibrio cholera* ghost-based vaccine, after it was identified as one of the conserved FP components (124). This vaccine gave rise to the induction of a prominent IgG2a response accompanied by a strong IFNγ cytokine production. However, in this case the combination of these favorable factors did not prevent the successful onset of parasitemia by *T. brucei* trypanosomes, as the infection was initiated with an extremely high number of 10^6 parasites.

By far the most often targeted trypanosome antigen is the tubulin protein, and as a variation on the idea, the actin protein (125–129, 135). Both proteins are part of the intracellular cytoskeleton structure and have functions in motility and intracellular organelle transport but are not accessible by the host antibody response. This makes the repeated reports of the successful induction of protection using various protocols very interesting. What all the protocols have in common, is that the waiting period between the last boost and the actual challenge was too short to asses vaccine induced memory, in some cases being limited to 6 days only. Hence, these protocols again assessed the direct effect of immune modulation by the procedure. What all reports also have in common is that none of them attempted to explain how the intracellular trypanosome proteins would have been targeted by the host antibody response in order to provide sterile protection. Once again, the most obvious explanation is that the applied protocol provided an improved inflammatory environment that favored the innate immune control of infection during the onset of parasitemia.

So far, most anti-trypanosome vaccine studied have been performed in mice, for obviously logistical reasons. Several authors have however questioned this approach and have argued that vaccine studies for AT should be conducted in the relevant host. The only molecular target that has been tested in this context is the *T. congolense* cysteine protease congopain, after it was shown that trypanotolerant N'Dama cattle, but not trypanosusceptible Boran cattle, mount and IgG1 response against this target (136). Successfully vaccinated cattle did show increased anti-CP serum antibody titers, as well as anti-VSG titers, coinciding with improved in parasitemia control (130). Next, the immunogenicity of CP was improved by coupling the catalytic domain of the enzyme to α_2-macroglobulin (137), and various adjuvants were compared leading to the adjuvant choice of Quil A^{TM} for future experiments (138). Important to stress here is that this approach is not an anti-parasite approach, but in the end, it is a way to modulate the host immune response and alleviate the inflammatory-associated immune pathology of trypanosomosis. The molecular mechanism by which this was achieved still remains open for discussion.

Using trypanosome enzymes as vaccine targets has also been applied in a *T. brucei* setting, targeting trans-sialidase (TS). Using TS encoding plasmid DNA, a single-dose vaccine experiment was followed 175 days later by a *T. b. brucei* GVR35 challenge, using 500 parasites. The obtained results show a 40% protection against infection for a period of at least 60 days (132). As the report did not detail a functional antibody analysis, it is difficult to provide a final mechanistic hypothesis for the partial protection. However, given the type of vaccine used (DNA vaccination) one could once again suggest that an IFNγ-driven helper response could be part of the equation, helping both the innate response immediately upon parasite challenge, as well as the IgG2a response that would occur upon B cell reactivation. In a follow-up vaccination approach against *T. congolense*, this time using recombinant TcoTS, protection levels reached 15–40% (131). Here, vaccine-induced memory responses were once again not addressed as the waiting period between the last boost and parasite challenged were limited to <14 days. Hence, this makes it very plausible that the limited level of protection observed was related in part to immune modulation and improved innate responses, rather than just anti-trypanosome antibody induction.

By considering the strategy of anti-pathology vaccination, rather than anti-trypanosome vaccination, our group has taken a radically different approach in the past to find new ways of dealing with the problems of trypanosomosis. After having identified the VSG-GPI anchor as the main driver of inflammatory pathology and macrophage-derived TNF production (13), we approached anti-disease vaccination for *T. b. brucei*, *T. evansi*, and *T. congolense* by using a liposome-based GPI vaccine. By challenging mice several times with GPI liposomes prior to trypanosome exposure, the infection-associated pathology was reduced and the lifespan of infected mice was significantly increased (133). Interestingly, using B-cell deficient mice, we were able to show that the observed effect was totally independent of antibody function, even though the protocol did result in the successful induction of antibodies in WT animals. These experiments conclusively showed that vaccine induced immune modulation of inflammation was at the core of the protection. The observation that the CD1d molecule was crucial for mediation of the observed protection, indicates a major role for the modulation of the innate immune system (133).

TABLE 2 | Summary of the detrimental effect of trypanosomosis in non-related vaccines, B cells, and B cell malignancies.

Parasite	Host	Disease/vaccine	Readout	Outcome	Functional readout	References
T. brucei	Mouse	*Bordetella pertussis*	CFU counts	Increased lung CFUs	Loss of vaccine protection	(82)
T. b. gambiense	Human	HIV	Ab titers Diagnostic test	Decrease in specificity	NA	(139)
T. b. gambiense	Human	Measles	Ab titers Diagnostic test	Decreased titers	NA	(140)
T. congolense/*T. vivax*	Cattle	CBPP	Ab titers/experimental infection	Decreased titers	50% susceptibility	(141)
T. congolense/*T. vivax*	Cattle/mice	Louping-ill virus	Ab titers	90% titer reduction	NA	(142)
T. congolense	Cattle	Foot-and-mouth virus	Ab titer	Decreased titers	Virus challenge/no effect on protection	(143)
T. congolense/*T. vivax*	Cattle	*Brucella abortus*	Ab titers	80–90% titer reduction (*T. congolense*)	NA	(144)
T. evansi	Buffalo	*Pastuerella multocida*	Ab titers/inflammation	Decreased titers	NA	(145)
T. evansi	Buffalo	*Pasteurella multocida*	Ab titers/lymphocyte proliferation	Decreased titers immuno-suppression	NA	(146)
T. evansi	Pig	Classic Swine Fever (CSF)	Ab titer/fever	Decreased titers	Decreased fever	(147)
T. brucei	Mouse	*Trichinella spiralis*	Ab titers/eosinophil counts lymphocyte proliferation	Decreased titers immuno-suppression	Decreased worm expulsion	(148)
T. brucei	Mouse	*T. brucei*/*homo-heterologous*	Parasite count	Short-lived specific protection	Loss of short-lived protection	(82)

NA, None applicable.

TRYPANOSOME INFECTIONS ABLATE THE EFFICACY OF NON-RELATED PROTECTIVE VACCINES

To date, it seems there no data is available that suggests that vaccine induced immunological memory can be recalled upon during ongoing trypanosome infections, in order to help the host to recover from infection. In addition, combined data shows that infection-associated inflammation is detrimental for the host immune system as whole, and as such could affect immune responses against non-related co-infections, or non-related vaccines. In recent years, this question has been addressed using both experimental mouse models and human case studies. In the past however, a significant amount of data has already been published indicating that trypanosomes do compromise the host immune system of natural hosts in general. In addition, there is data available showing that HAT and AT affect antibody titers against non-related disease, which is particularly important when considering antibody-based diagnostic test evaluation in trypanosomosis endemic areas. All these observations have been summarized in **Table 2**.

The first time that the detrimental effect of experimental mouse trypanosomosis was investigated in detail in a heterologous vaccine setting, was by analyzing the effect of *T. b. brucei* infections on the efficacy of the commercially available DTPa vaccine (Boostrix®), which provides protection against diphtheria, tetanus, and whopping caught infections (83). After having observed a complete and permanent ablation of vaccine-mediated protection by the trypanosome, it was proposed that it should be assessed whether such immune destructive effect could threaten vaccine efficacy in humans. While experimental pathogen re-challenge experiments in humans can obviously not be performed in a HAT setting, two follow-up observations are worth mentioning in this context. First, it was shown that *T. b. gambiense* infections decrease the specificity of antibody detection tests for HIV diagnosis, warning that classic algorithms that are being used for test interpretation on non-HAT individuals, might not be able to provide adequate diagnostic answers in HAT patients, even after treatment (139). Next, it was shown that HAT patients who had been vaccinated against measles had significantly lower antibody serum titers compared to non-HAT individuals. Antibody titers remained low after curative anti-HAT treatment, although they were above the guideline cut-off in healthy individuals (140). However, as was the case for the HIV diagnostics, the measured antibody levels could very well be an over estimation of the actual functional titer. This is also what has been observed in the case of contagious bovine pleuropneumonia (CBPP) vaccinations in cattle. Here it was shown that in *T. congolense* infected animals, a vaccine against CBPP lost 50% of its efficacy, while only a minor drop in antibody titers was observed (141). Such drop in non-trypanosome related vaccine efficacy, due to the presence of animal trypanosomosis, has been observed in models for louping-ill virus protection (142), foot-and-mouth

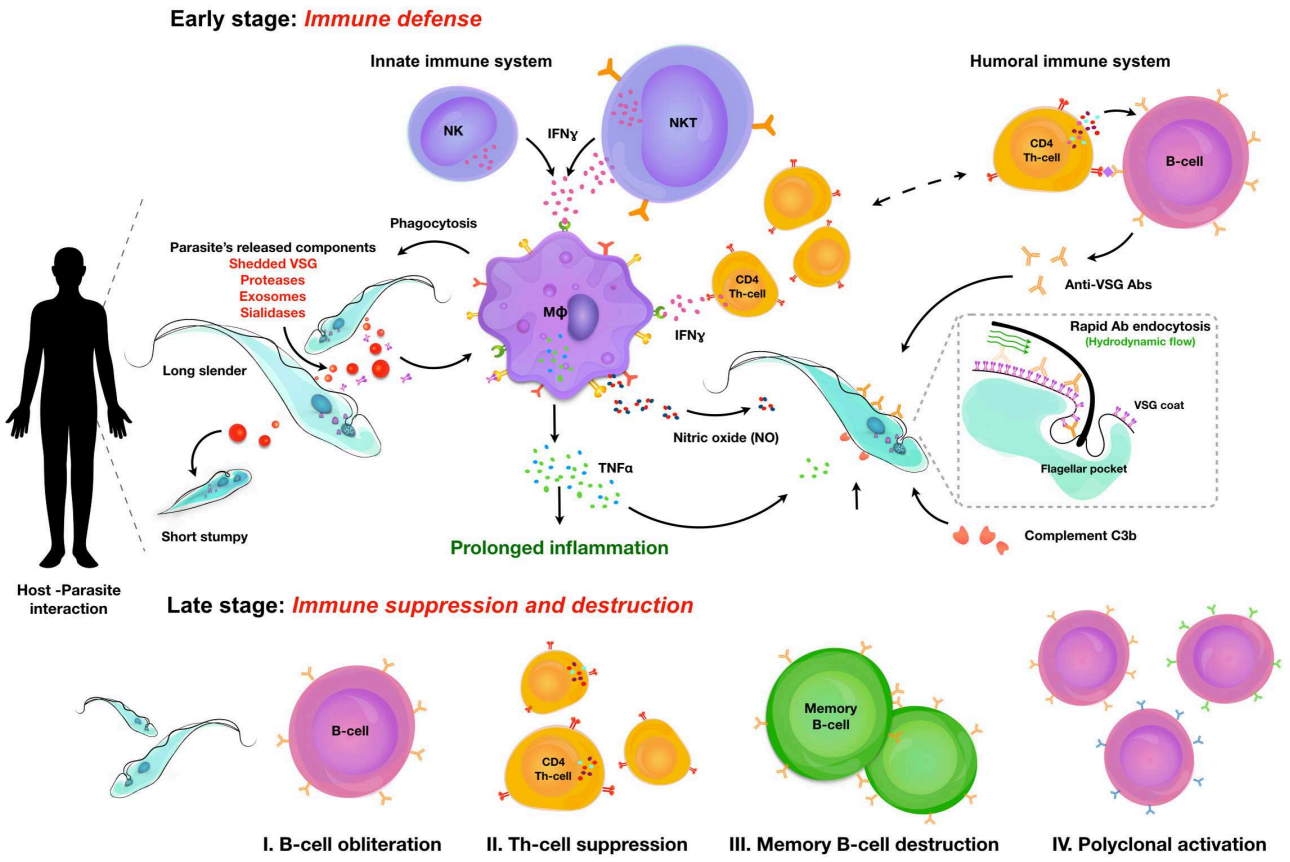

FIGURE 1 | Overview of the crucial aspects of trypanosome-host interactions. Upon infection, trypanosomes are confronted with an early stage inflammatory immune response in which IFNγ is being produced by NK, NKT and subsequently $CD4^+$ T cells. This drives the activation of macrophages which in term respond to parasite secreted and released factors, including shedded VSG (Variant Surface Glycoprotein). Activated macrophages produce TNF and Nitric Oxide (NO) that negatively impact on parasite fitness. Antibodies, derived first through T cell independent B cell activation and later through T cell dependent activation, will attack the surface of the trypanosome. Deposition of the C3b complement will also occur. Together, these immune molecules will help in controlling parasitemia, but trypanosomes themselves have defense mechanisms that will help survival. Through lateral VSG movement to the flagellar pocket (FP) antibodies and complement factors are cleared, limiting damage. In order to prevent early killing of the host through parasite overpopulation, trypanosomes have developed quorum sensing, which results in density dependent growth arrest and preparation for vector transmission. Combined, this innate control of infection results in prolonged inflammation with a detrimental outcome for the adaptive immune response. Late stage infections are characterized by B cell destruction, T cell mediated immune suppression, loss of B cell memory recall capacity and irrelevant polyclonal B cell activation.

vaccination (143), a cattle vaccine model for *Brucella abortus* (144), immunization against *Pasteurella multocida* (145, 146), classic swine fever (CSF) vaccination (147), and in a vaccine setting for *Trichinella spiralis* (148). Finally, staying within the boundaries of the trypanosome mouse model, we assessed functional anti-VSG memory responses. Here we used a live infection model rather than a vaccine model in which mice were first challenged with low-virulent parasite clone, followed by a secondary infection with a high virulent clone expressing either an identical VSG surface coat is the parasite used in the primary infection (homologous challenge) or a different VSG coat (heterologous challenge). The obtained result showed that while the first infection did provide VSG-specific protection against a homologs challenge, the protection was short-lived and disappeared within 10 days after clearance of the first wave of the primary infection. Using B cell knock-out mice, we showed the temporary protective response relied on T-cell independent antibodies (82). However, by using a battery of cytokine deficient mice including TNF and IFNγ knock-out mice it was obvious that the short-lived protective antibody response only works in an inflammatory environment that is driven by the early innate immune response. This can explain the abolishment of the protection by day 17 post infection, despite the presence of high anti-VSG antibody titers at the time of challenge.

CONCLUSIONS

Extracellular trypanosomes are very successful parasites that today are still expanding their territory. While the terminology "African trypanosomes" is often used synonymously for salivarian trypanosomes, it should be remembered that *T. evansi* was the first extracellular trypanosomes to be discovered, and that this discovery was made in India. Hence, the presence of

these parasites should be considered as a global issue, with a possibility of affecting all commercially important livestock mammals as well as game animals. Due to their extracellular nature, and their continuous confrontation by the mammalian immune system, trypanosomes had to acquire multiple defense mechanisms to overcome mainly the dangers posed by host antibodies (**Figure 1**). Coating their extracellular membrane with a dense layer of VSG glycoproteins offers protection against antibodies, but most likely not in ways that have been suggested in the early days of trypanosome research. First, when during infection the host manages to mount an anti-VSG response, binding immunoglobulins function as molecular sails, and complexed membrane VSG molecules are rapidly transported to the flagellar pocket where they are endocytosed, cleaned and recycled or replaced. Having a *de facto* unlimited library of VSG genes, pseudogenes and the capacity of generating mosaic genes, allows the parasite to switch the antigenic type of the coat and hence avoid complete elimination of the population. Combining this mechanism with the destruction of the host B cell compartment allows the trypanosome to outrun the dangers posed by the host humoral immune response. In addition, multiple studies using mouse knock-out technology, natural mutant mouse strains and AT as well as HAT observations have shown that complement-mediated lysis cannot be used as part of the host defense system, as the complement cascade is blocked from forming C5–C9 components needed for actual lysis. This leaves the host with the system of iC3b-mediated phagocytosis, mainly performed by inflammation-activated Kuppfer cells. In order to protect itself from phagocytosis, the parasite once again uses VSG recycling to prevent total population elimination by clearing iC3b surface deposition. Important however in the case of a parasite, is the fact that killing the host is not a good survival strategy. Hence, trypanosomes have developed a system of quorum sensing that allows them to regulate proliferation in response to parasite density, and most likely is also capable of sensing excessive infection-associated inflammatory tissue damage. While this is crucial to ensure prolonged host survival, it does not prevent the infection of leading to the virtually complete destruction of the host B cell compartment. The latter is most likely the reason for the fact that so far not a single promising laboratory vaccine has been translated into a useful field application. In addition, it explains why many commercial vaccines against unrelated diseases appear to lack efficacy in trypanosome endemic areas. The latter is the case for trypanosusceptible animals, and this could be the foundation for the distinction between susceptibility and tolerance for infection. While trypanotolerance has been described in detail for particular cattle breeds, it is only recently that this phenomenon has now been suggested to also occur in some humans. It would be interesting to see in the future how the B cell compartment of such individuals behaves, as compared to the bulk of the human population that is considered trypanosusceptible. Subsequently, it could be assessed in a HAT context how the relative importance of the innate immune system and the adaptive immune system compare to each other, both in the control of onset of infection as well as in the control of the chronic infection stage.

AUTHOR CONTRIBUTIONS

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Notch Signaling Pathway Expression in the Skin of Leprosy Patients: Association With Skin and Neural Damage

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Introduction: Leprosy is an infectious disease caused by *Mycobacterium leprae*, a debilitating disease that affects the skin and peripheral nerves. It is possible that tissue changes during infection with leprosy are related to alterations in the activity of the Notch signaling pathway, an innate signaling pathway in the physiology of the skin and peripheral nerves.

Methods: This is a descriptive observational study. Thirty skin biopsies from leprosy patients and 15 from individuals with no history of this disease were evaluated. In these samples, gene expressions of cellular components associated with the Notch signaling pathway, *Hes-1, Hey-1, Runx-1 Jagged-1, Notch-1*, and *Numb*, were evaluated using q-PCR, and protein expression was evaluated using immunohistochemistry of Runx-1 and Hes-1.

Results: Changes were observed in the transcription of Notch signaling pathway components; Hes-1 was downregulated and Runx-1 upregulated in the skin of infected patients. These results were confirmed by immunohistochemistry, where reduction of Hes-1 expression was found in the epidermis, eccrine glands, and hair follicles. Increased expression of Runx-1 was found in inflammatory cells in the dermis of infected patients; however, it is not related to tissue changes. With these results, a multivariate analysis was performed to determine the causes of transcription factor Hes-1 reduction. It was concluded that tissue inflammation was the main cause.

Conclusions: The tissue changes found in the skin of infected patients could be associated with a reduction in the expression of Hes-1, a situation that would promote the survival and proliferation of *M. leprae* in this tissue.

Keywords: *Mycobacterium leprae*, Hes-1 transcription factor, Runx-1 transcription factor, inflammation, epidermis, hair follicle, eccrine gland, nerve fiber

INTRODUCTION

Leprosy is a neglected infectious disease caused by *Mycobacterium leprae*, an obligate intracellular microorganism that cannot be cultivated in axenic media (1). In addition, this mycobacterium is characterized by a marked affinity for the skin and peripheral nerve trunks and is responsible for the disability of more than four million people worldwide (2).

This disease has accompanied humanity for millennia, and it is a public health issue in some tropical countries, despite elimination efforts by the World Health Organization (WHO) (3). One of these efforts is the Global Strategy 2016–2020, which aims to reduce disability in children diagnosed with leprosy to zero and to reduce disability in new cases to less than one per million inhabitants (4). As published by Blok et al. (5), who used a simulated model to predict the future incidence of this disease in high-burden countries, it became clear that it is unlikely these WHO targets will be reached by 2020 unless additional measures are taken.

One measure that in the future could have an impact on reducing the burden and disability generated by *M. leprae* is to deepen our understanding of the physiopathogenic mechanisms related to disability and the proliferation of this mycobacterium in the host, from which tools can be generated to facilitate its detection and control. One of these mechanisms could be related to changes in the activation of the Notch signaling pathway, which is an innate cellular component involved in multiple processes of cellular proliferation and differentiation at the levels of the skin and peripheral nerves (6, 7).

In the nerve, our hypothesis is that Notch activation could be linked to the neural damage model proposed by Tapinos et al. (8), in which *M. leprae* induces the overexpression of cyclin D1 in the Schwann cell (SC), which generates dedifferentiation and demyelination of the nerve fiber. The association between cyclin D1 and Notch is plausible since, in other cellular models, it has been observed that cyclin D1 alone is not capable of generating changes at the cellular level. Indeed, changes are promoted through the activation of Notch (9) and the association of these two cellular components during infection. This indicates that the neural damage promoted by *M. leprae* could be linked to that reported by Woodhoo *et al.* (7), who observed that the postnatal activation of Notch induces the demyelination of the nerve fiber. Given that, the transcription factors of the Hes family, Hey, and even Runx-1 behave as repressors of the genes in charge of maintaining myelin in the nerve (10).

In the skin, we propose that *M.* leprae-induced modulation of Notch signaling pathway components - including Notch-1 receptor, Jagged-1 ligand, transcription factors Hes-1, Hey-1, Runx-1, and the Numb protein - could compromise the differentiation of cutaneous immune cells (11) and stem cells and even promote the demyelination of nerve fibers (10), facilitating the survival and proliferation of *M. leprae* and the manifestation of disabilities in the host.

Therefore, the aim of this study was to establish the relationship between the expression of components of the Notch signaling pathway (Hes-1, Hey-1, Runx-1, Jagged-1, Notch-1, and Numb) with tissue changes in the skin and dermal nerve fibers of leprosy patients.

MATERIALS AND METHODS

Type of Study

A descriptive observational study was conducted with cross-sectional analysis.

Sample Collection

Thirty skin samples were obtained from patients with multibacillary leprosy (MB) and active skin lesions (erythematosus + sensory compromise). These were collected in the departments of Santander, Norte de Santander, Boyacá, and Antioquia. The inclusion criterion for leprosy patients was that they had a clinical diagnosis of MB leprosy confirmed by bacteriological or histopathological examination. In addition, 15 skin samples were collected from discarded tissue resulting from plastic surgery from volunteer individuals with no personal or family history of leprosy.

Prior to the collection of these tissues, all participants gave their informed, written consent, which was endorsed by the institutional ethics committee for human research at CES University (Act No. 101 of 3 March 2017).

Gene Transcription Analysis

RNA Extraction and Real-Time PCR (qPCR)

Skin samples were stored in RNAlater (Cat. No. 76104, Qiagen) at −20°C until RNA extraction. Total RNA was extracted using the RNeasy Mini Kit (Cat. No. 74104, Qiagen) following the manufacturer's instructions, and was eluted in 30 μL RNase- and DNase-free water (12). RNA concentration and purity were determined by spectrophotometry using a Nanodrop 1000 (Nanodrop Technologies, Wilmington, USA) and stored at −80°C until reverse transcription was performed.

RNA samples were reverse transcribed to create cDNA using RT^2 First Strand Kit (Cat. No. 330404, Qiagen), following the manufacturer's instructions (13). RT-PCR was performed with a C1000TM thermocycler (Bio-Rad, Hercules, Ca, USA.)

qPCR reactions were carried out on a Custom RT^2 PCR array (Cat. No. 33171, Qiagen) for the following genes: *Hes-1, Hey-1, Runx-1, Jagged-1, Notch-1,* and *Numb*. To each well of this array, 20 μL master mix prepared for each skin sample was added: 11.5 μL RT^2 SYBR Green ROX FAST master mix (Cat. No. 330622; Qiagen), 1.5 μL cDNA, and 10 μL RNase-and DNase-free water. The conditions of the qPCR included a heating step for the activation of the Taq polymerase for 10 min to 95°C, followed by 40 amplification cycles (15 s at 95°C, and 30 s at 60°C). A dissociation or melting curve from 70°C to 90°C, with temperature increments of 0.5°C, was then plotted to confirm the amplification of the gene of interest (13). All qPCR reactions were carried out in a Corbett Rotor-Gene® 6000 thermocycler (Qiagen, Valencia, Ca, USA.).

The Ct (cycle threshold) values were recorded in an Excel spreadsheet and loaded into a software program provided by the manufacturer (Qiagen) for data analysis. Ct values were classified (leprosy and non-leprosy) and standardized using Ct averages from three housekeeping genes (GAPDH, RPLP0, and ACTB). Changes in gene expression were analyzed using the $2^{-\Delta\Delta Ct}$ method (14).

Histopathological Analysis

Tissue Preparation for Histopathological Study

A fragment of the skin samples was fixed in 10% formalin blocking solution and paraffinized for the purposes of histopathological and immunohistochemical (IHC) evaluation. For histopathological evaluation, the paraffinized tissue was cut into 4–5-μm-thick sections, stained with hematoxylin and eosin (H&E) stain, and examined to confirm whether the tissue corresponded to healthy or *M. leprae*-infected skin. Histopathological evaluations were conducted by an expert dermatopathologist.

H&E Analysis of Tissue Sections

In H&E-stained tissue sections, the following histopathological patterns were evaluated: trophic changes in the epidermis, location and severity of the inflammatory infiltrate, presence of granulomas and necrosis, and periadnexal and subcutaneous cellular tissue involvement (15). The information was recorded in a format designed for this purpose. There is a description of H&E staining in **Supplementary Methods**.

Immunohistochemical Stain (IHC)

Skin tissue sections of 4 μm were deparaffinized in an oven at 58°C overnight, followed by three 5-min xylol immersions. After deparaffinization, the tissue was rehydrated using isopropanol solution dehydrated in absolute alcohol. The tissue sections were then blocked in a 6% hydrogen peroxide solution for 5 min, and epitope recovery was induced by heat in a water bath at 98°C and was performed with EDTA 10 mM.

After the antigenic recovery, the primary antibodies anti-Hes-1 (Cat. No. ab119776, monoclonal, dilution 1:100, Abcam), anti-Runx-1 (Cat. No. ab35962, polyclonal, dilution 1:250, Abcam), anti-S-100 (Cat. No. Z0311, polyclonal, dilution 1:5600, Dako), anti-CD68 (Cat. M0876, monoclonal, dilution 1:600, Dako), and anti-cyclin D1 (Cat. EP12, Master diagnostics) were added to each tissue section for each IHC assay. Each antibody dilution was performed in 1% bovine serum albumin (BSA) in 1X TBS (**Table S1**) then incubated for 1 h at room temperature. The primary antibody signal was then amplified with Quanto Amplifier (Cat. TL-125-QHL Thermo Fisher Scientific) for 10 min. After this, the secondary antibody—which is a specific polymer for anti-mouse and anti-rabbit IgG from the UltraVision™ Quanto Detection System HRP DAB (Cat. No. TL-125-QHL Thermo Fisher Scientific)—was incubated for 10 min according to the manufacturer's instructions. Staining patterns were developed using a 3.3' diaminobenzidine (DAB) solution for 3 min. Tissue sections were contrasted with hematoxylin, clarified with ammoniacal alcohol, and hydrated in pure isopropanol. As a negative control, the primary antibody was replaced by 1% BSA **Figure S1**. In addition, positive controls are reported in **Table S1** and can be viewed in **Figure S2**. Finally, the specificity of the primary antibodies related to the Notch signaling pathway used in this investigation was confirmed through Western blot **Figure S3** and **Supplementary Methods**).

Immunohistochemical Analysis

The stained tissue sections were evaluated using two types of analysis, as follows.

Morphometric analysis was used in the regions of interest lacking inflammatory cell infiltrates. Briefly, the digital images were acquired using an optical microscope (Nikon Eclipse E200), captured under 40x magnification with a Lumenera (Infinity1) camera and stored in a TIFF (Tagged Image File Format) graphic format. Subsequently, 135 images were transferred to the free ImageJ version 1.52p software (NIH, Bethesda, Maryland), each having 2048 × 1536 pixels. In this software, the images were processed using the tool "Color Deconvolution," which is an add-on that allows the breakdown of RGB color images and separates them into three complementary images (16). The first image corresponds to contrast staining with hematoxylin (R 0.65, G 0.704, B 0.286). The second image corresponds to the stain performed with the chromogen-DAB (R 0.268, G 0.570, B 0.776), and the third is a residual type image (R 0.711, G 0.423, B 0.561).

The supplementary images, corresponding to the staining with hematoxylin and DAB, were later converted to grayscale (8 bits) to create binary images possessing two pixel values, 255 pixels (white) and zero pixels (black) (17). Using the "threshold" command, an average threshold of 100 pixels was established on a histogram expanded for white color and zero pixels for black color in each of the evaluated images, which allowed for proper identification of the nuclei stained with DAB and hematoxylin. Finally, the command "analyze particles," which scans the threshold image and measures the cells covered in accordance with the predefined parameters, was selected. In this way, the total number of cells stained with DAB and hematoxylin and the percentage of chromogen staining were determined. To validate the results of the morphometric analysis, a visual analysis in the areas of interest was carried out by an expert dermatopathologist, who was not involved with the ImageJ evaluation **Tables S2, S3.**

Visual analysis (semi-quantitative) was conducted by an expert dermatopathologist, using a visual score and carried out on skin structures compromised by inflammatory infiltrate. It is relevant to mention that this visual measurement was intended to reduce any information bias that could be generated in the digital analysis of these infiltrates into the tissue. Briefly, visual analysis was performed with an optical microscope (Leica dm500), using a magnification of 10x to 40x. This analysis was conducted under the guidelines of the College of American Pathologists, and the parameter chosen for this evaluation was the percentage of cells positive for each of the markers (18, 19). The visual score for the percentage of positive cells is, $0 \leq 1\%$, $1 = 1–25\%$, $2 = 25–75\%$, $3 \geq 75\%$.

The staining of skin nerve fibers marked with anti-S-100 was classified using the pathological patterns described in **Table S4**.

Bacillary Index (BI) and IgM anti-NDO LID Serology

A description of these tests is available in the section **Supplementary Methods**.

Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences, version 21 (SPSS). The univariate analysis for qualitative variables was performed through the calculation of absolute and relative frequencies. In quantitative variables, measures of central tendencies (mean and median) were calculated. In addition, the normality of these variables was determined by the Shapiro-Wilk test. The bivariate analysis for qualitative variables was performed through Pearson's chi-square test, and the analysis of qualitative-quantitative variables was performed using the Mann-Whitney *U*-test or Student's *t*-test. The multivariate analysis was performed using a multinomial logistical analysis. The significance of the *p*-value was set to <0.05 for all analyses performed.

RESULTS

Sociodemographic and Clinical Characteristics of Participants

Thirty patients with leprosy (90% male and 10% female), with a median age of 51 years, were evaluated. Using the WHO standard, 100% of the patients were classified as multibacillary, and 96.6% persisted with a positive BI. According to the Ripley-Jopling classification, 66.7% presented with lepromatous leprosy, 30% with borderline lepromatous leprosy, and 3.3% with borderline tuberculoid leprosy. In assessing the degree of disability, we found that 16.7% of the leprosy patients presented with Grade 0, 46.7% with Grade 1, and 36.6% with Grade 2; it is relevant to mention that 56.7% of patients had a history of having suffered from a type-II leprosy reaction, while the remaining 43.3% had not presented these immunological categories. In addition, 86.6% of patients were seropositive for IgM anti-NDO-LID (**Table 1**).

With regard to the sociodemographic characteristics of the 15 non-leprosy individuals, 60% were female and 40% male, with an average age of 42.8 years, and none had any personal or family history of leprosy (**Table S5**).

TABLE 1 | Sociodemographic and clinical characteristic of the leprosy patients.

Characteristic of the leprosy patients evaluated		*n* = 30 (%)
Sex	M	27 (90%)
	F	3 (10%)
Median age* (Range)		51 (28–65)
Geographic area	Santander (Norte y Sur)	20 (66.6%)
	Antioquia	4 (13.4%)
	Boyacá	6 (20%)
WHO classification	PB	0
	MB	30 (100%)
R&J classification	BT	1 (3.3%)
	BL	9 (30%)
	LL	20 (66.7%)
Grade disability	0	5 (16.7%)
	1	14 (46.7%)
	2	11 (36.6%)
History of leprosy reactions	No	13 (43.3%)
	LR1	0
	LR2	17 (56.7%)
Bacillary index	(+)	29 (96.6%)
	(-)	1 (3.4%)
IgM anti-NDO-LID	(+)	26 (86.6%)
	(-)	4 (13.4%)

Histological and Histopathological Characteristics

Histopathological findings for leprosy skin: Atrophic changes were observed in the epidermis of 73.3% of patients. The skin of

TABLE 2 | Histological and histopathological description.

Histopathological and histological description			
Variables		Leprosy skin *n* = 30 (%)	Non-leprosy skin *n* = 15 (%)
Epidermal atrophy	Yes	22 (73.3%)	0
	No	8 (26.7%)	15 (100%)
Epidermal Hyperplasia	Yes	3 (10%)	0
	No	27 (90%)	15 (100%)
Inflammatory infiltrate	No infiltrate	1 (3.3%)	15 (100%)
	Perivascular	15 (50%)	0
	Nodular	4 (13.3%)	0
	Diffuse	10 (33.4%)	0
Granuloma	Non-granuloma	7 (23.3%)	15 (100%)
	Epithelioid	13 (43.3%)	0
	Suppurative	10 (33.4%)	0
Necrosis	Yes	2 (6.6%)	0
	No	28 (93.4%)	15 (100%)
Periadnexal infiltrate	No infiltrate	5 (16.7%)	15 (100%)
	Pilosebaceous unit	1 (3.3%)	0
	Perieccrine	12 (40%)	0
	Mixed (pilosebaceous-perieccrine)	12 (40%)	0
Compromised subcutaneous cellular tissue	No compromise	15 (50%)	15 (100%)
	Lobular	10 (33.4%)	0
	Septal	0	0
	Mixed (lobular-septal)	5 (16.6%)	0
Morphology of dermal nerves	Intact	1 (3.3%)	15 (100%)
	Infiltrated	13 (43.4%)	0
	Fragmented	1 (3.3%)	0
	Absent/destroyed	15 (50%)	0

TABLE 3 | Changes in the expression of some components of the Notch signaling pathway in leprosy skin vs. non-leprosy skin.

Gen	Upregulated or Downregulated	U Mann-Whitney *p*-value
Hes-1	**-2.32**	**<0.0001**
Runx-1	**3.69**	**0.043**
Jagged-1	−1.73	**0.009**
Notch-1	−1.40	0.08
Hey-1	1.56	0.79
Numb	1.31	0.67

The bold values are showing statistical significance.

96.7% of patients had some type of inflammatory infiltrate, and 76.7% presented granulomas.

At the level of the skin annexes, 83.3% showed alterations, 40% with infiltrate at the peri-eccrine level, 3.3% in the pilosebaceous unit, and 40% had mixed-type infiltrate (of the eccrine glands and in the pilosebaceous unit). With regard to subcutaneous cellular tissue, it was impaired in half of the leprosy patients, with a lobulillar-type impairment in 10% and mixed (lobulillar-septal) in 40%. It is also relevant to mention that areas of cutaneous necrosis were found in only 6.6% of patients. In addition, in the evaluation of dermal nerves of leprosy patients, trophic changes were observed in 96.7% of samples, showing absence in 50%, infiltrates in 43.4%, and fragmentation in 3.3% (**Table 2**).

Histological findings for the skin samples of non-leprosy individuals: In all samples, no changes were observed, either trophic, inflammatory, or nervous, in the evaluated structures (**Table 2**).

When comparing the histopathological changes of leprosy and non-leprosy samples, a statistical difference was observed (**Table S6**).

Differences in the Expression of Notch Signaling Pathway Components in Skin Samples of Leprosy Patients and Non-leprosy Individuals

To establish whether there are changes in the expression of genes of interest (*Hes-1, Hey-1, Runx-1, Numb, Notch-1,* and *Jagged-1*), a dispersion chart was plotted. We found evident changes in the expression of two of the evaluated genes: *Hes-1*, downregulated 2.32 times, and *Runx-1*, upregulated 3.69 times in leprosy skin samples (**Table 3**, **Figure 1A**).

When comparing gene expression quantification via the delta-delta Ct method, significant changes were noted in the expression of *Hes-1* ($p < 0.0001$), *Runx-1* ($p = 0.043$), and *Jagged-1* ($p = 0.009$) (**Table 3**, **Figure 1B**, and **Figure S4**). To confirm these changes and to characterize this difference in a clearer manner, a heat map was made, which provided a graphical and coded representation of colors related to the expression of these genes (green = reduced expression, red = increased expression). *Hes-1* was found to have reduced expression in 83.3% of the leprosy samples and increased expression in 67% of non-leprosy samples. *Runx-1* expression was increased in 33.3% of leprosy skin samples and in 100% of non-leprosy samples. *Jagged-1* was reduced in 83.3% of leprosy samples and increased in 53.3% of non-leprosy samples (**Figure 1C**).

Identification of Runx-1 Expression in Cutaneous Structures of Leprosy Patients and Non-leprosy Individuals

We established that Runx-1 transcription factor is not expressed in the dermal nerves of leprosy patients (**Figure 2**). On the other hand, immunostaining showed that the overexpression of Runx-1 in leprosy patients was due to its expression in the cells present in the dermal inflammatory infiltrate. The dermis showed a median of 98.5 cells stained for Runx-1 per field observed in leprosy patients (interquartile range (IQR) = 44.7–177 cells per field), while the median was only 4 cells per field in non-leprosy patients (IQR= 1.5–5.5 cells per field) ($p < 0.0001$) (**Figures 3A–E**). Later, we established using CD68 marker that the cells stained for Runx-1 correspond to macrophages that are generally distributed diffusely at the cutaneous layer level (**Figures 3F–G**).

Other skin structures in which Runx-1 marking was observed were the skin annexes (eccrine glands, hair follicles). At the level of the eccrine glands, a percentage of Runx-1-positive cells greater than 75% was observed in 63.3% of the eccrine structures of leprosy samples and in 73.3% of those of non-leprosy samples, a non-significant difference ($p > 0.05$). In the visualized hair follicles, the percentage of Runx-1-positive cells was greater than 75% in 50% of the hair follicles of leprosy samples and in 75% of non-leprosy samples, a slight but non-significant ($p > 0.05$) reduction in leprosy samples (**Table 4**, **Figure 4**).

Identification of Hes-1 Expression in Cutaneous Structures of Leprosy Patients and Non-leprosy Individuals

IHC of Hes-1 confirmed changes in expression in three skin structures of leprosy patients: epidermis, eccrine glands, and hair follicles. In the epidermis, a median of 4.2% of Hes-1-marked cells was observed in leprosy samples (IQR = 1.5–7.1 stained cells per field), while in non-leprosy samples it was 73.8% (IQR = 45.6–77 stained cells per field) ($p < 0.0001$) (**Figures 5A–C**).

At the level of the skin annexes, we also showed differences in the expression of Hes-1 when comparing leprosy and non-leprosy samples. In the eccrine glands, we observed that in 80% of leprosy samples, the percentage of Hes-1-positive cells ranged from <1% to 25%, while in 93.4% of non-leprosy samples, the percentage of Hes-1-positive cells was greater than 75% ($p < 0.0001$) (**Figures 5D,E**, **Table 5**). With respect to hair follicles, 83.3% of the follicles visualized in leprosy samples showed a percentage of Hes-1-positive cells ranging from <1% to 25%, while in 100% of the follicles evaluated in non-leprosy samples the percentage was greater than 75% ($p < 0.0001$) (**Figures 5F,G**, **Table 5**).

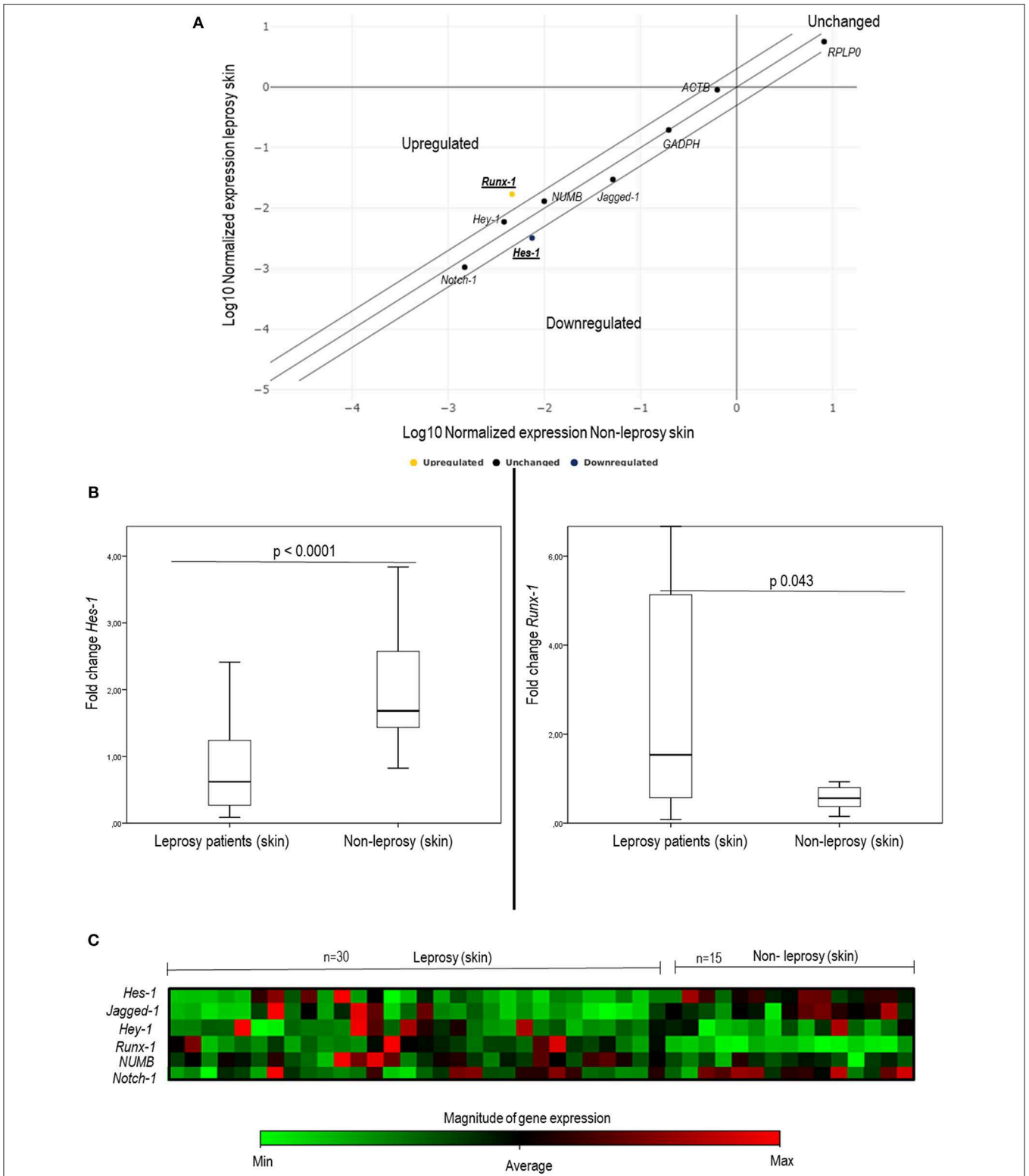

FIGURE 1 | Changes in the expression of some components of the Notch signaling pathway in leprosy skin vs. non-leprosy skin using q-PCR. **(A)** The dispersion chart shows non-significative changes in genic expression (≤ 2) for *Notch-1, Jagged-1, Hey-1,* and *Numb* (black dots). Outside of the matrix, *Hes-1* and *Runx-1* show significant change (≥ 2), and there are significant changes in gene expression (≥2) for *Hes-1* (Blue dot - downregulated) and Runx-1 (yellow dot - upregulated). **(B)** Whisker box charts show lower expression of *Hes-1* and an increase in the expression of *Runx-1* in leprosy patients vs. non-leprosy ($p < 0.05$). **(C)** Heat map shows the gene expression of *Hes-1, Runx-1, Notch-1, Jagged-1, Hey-1,* and *Numb* in leprosy and non-leprosy patients. Green tones indicate a downregulated gene, and red tones indicate upregulated genes.

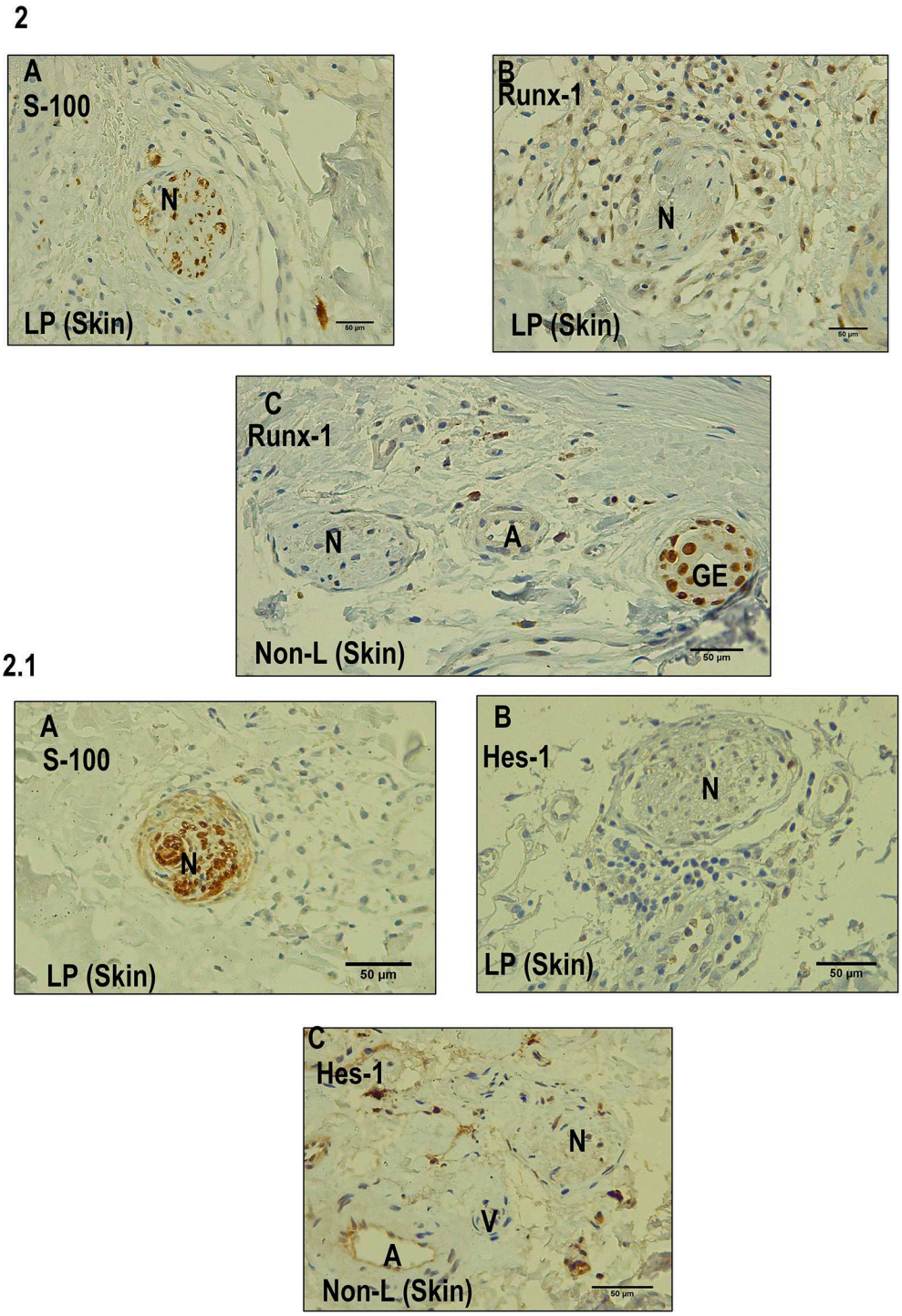

FIGURE 2 | Immunohistochemistry with Runx-1 and Hes-1 in dermal nerves (leprosy skin and non-leprosy skin). **(A)** Dermal nerve (N) stained with S-100 in a leprosy sample (LP). **(B)** Dermal nerve (N) in a leprosy patient (LP) negative for Runx-1. **(C)** Dermal nerve (N) 1 in a non-leprosy sample (non-LP) negative for Runx-1. A: artery, EG: eccrine glands. All the figures have a 50-µm scale bar. **2.1 (A)** A dermal nerve (N) stained with S-100 in a leprosy sample (LP). **(B)** Dermal nerve (N) in leprosy sample (LP) negative for Hes-1. **(C)** Dermal nerve in a sample non-leprosy (Non-LP) negative for Hes-1. A: artery, V: venule, N: nerve. All the figures have a 50-µm scale bar.

On the other hand, the IHC analysis of Hes-1 allowed us to rule out that this transcription factor is being expressed in the dermal nerve fibers of leprosy patients (**Figure 2A**). Finally, the IHC findings of Hes-1 were validated through Western blot, showing a significant reduction in the expression of Hes-1 in the skin samples of leprosy patients ($p < 0.05$) (**Figures 5H,I**).

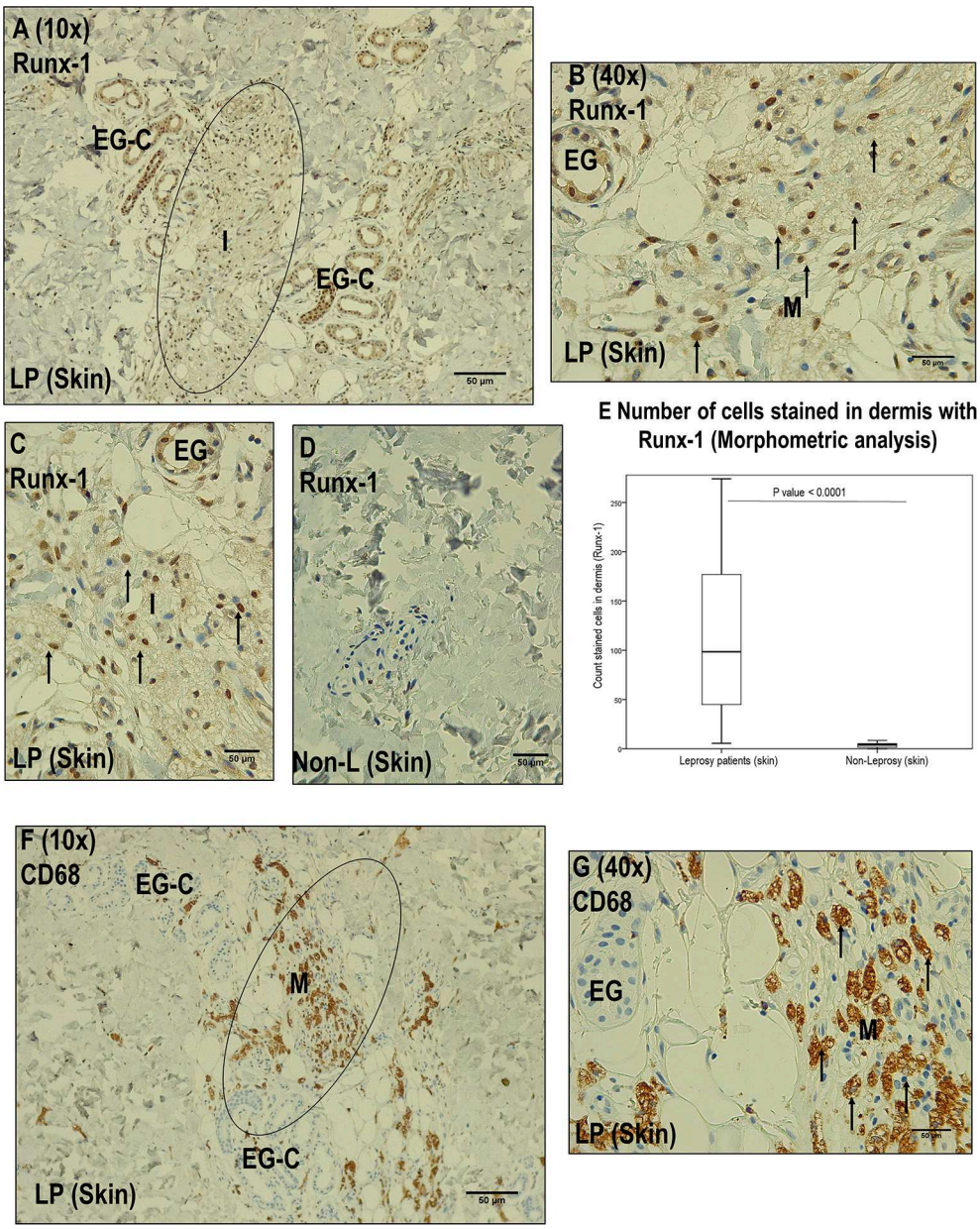

FIGURE 3 | Immunohistochemistry with Runx-1 in leprosy skin vs. non-leprosy skin**. (A)** Image (10x magnification) showing leprosy dermis (LP), with inflammatory cells stained with Runx-1 (I) surrounding eccrine glands. **(B)** Image (40X magnification) showing a zone marked in **(A)** showing positive Runx-1 stained cells (macrophages, M). **(C)** Dermic inflammatory infiltrated sample (I) stained with Runx-1. **(D)** Non-leprosy dermic sample (Non-LP) negative for Runx-1. **(E)** Comparison of the Runx-1 stained cells in leprosy vs. non-leprosy skin samples ($P < 0.05$). **(F)** Image (10x magnification) showing a leprosy skin sample (LP) stained with CD68 to confirm the presence of macrophages (M) in the inflammatory infiltrate (I) surrounding eccrine glands. **(G)** Image (40X magnification) showing the zone marked in **(F)** showing macrophages (M). All the figures have a 50-µm scale bar.

Histopathological and Gene Findings That Might Explain the Changes in the Expression of Hes-1 (Multivariate Analysis)

After establishing the dermal structures in which changes in the expression of Hes-1 were observed (epidermis, hair follicle, and eccrine glands), we decided to evaluate how the inflammatory, trophic, neural, and genetic changes found in the tissue could explain or be related to the reduction of Hes-1. For this, we performed a multinomial logistic analysis relating to the percentage of Hes-1-positive cells in these structures with gene findings (*Jagged-1, Notch-1*) and histopathological findings (trophic changes, inflammatory changes, and involvement of dermal nerves) in which a difference was found between leprosy and non-leprosy samples (**Table 3**, **Table S3**).

When relating these findings to the percentage of Hes-1-positive cells, we observed that changes in the expression in the epidermis, eccrine glands, and hair follicles was mainly related to inflammation of the cutaneous tissue ($p < 0.0001$). In addition, at the epidermis, eccrine glands, and hair follicles, the reduction of Hes-1 was also related to a lower expression of the Jagged-1 ligand ($p < 0.05$) (**Table 6**).

TABLE 4 | Differences in the expression of Runx-1 in eccrine glands and hair follicle between leprosy skin and non-leprosy skin.

Sample		Leprosy patients (Skin)	Non-leprosy (Skin)	Chi-square *p*-value
		***n* = 30 (%)**	***n* = 15 (%)**	
Stained cells in eccrine glands % (Runx-1)	< 1%	2 (6.6%)	1 (6.6)	0.648
	1-25%	3 (10%)	0	
	25-75%	6 (20%)	3 (20%)	
	> 75%	19 (63.3%)	11 (73.3%)	
		***n* = 22 (%)**	***n* = 12 (%)**	
Stained cells in hair follicle % (Runx-1)	< 1%	1 (4.5%)	0	0.480
	1-25%	1 (4.5%)	0	
	25-75%	9 (41%)	3 (25%)	
	> 75%	11 (50%)	9 (75%)	

TABLE 5 | Differences in the expression of Hes-1 in eccrine glands and hair follicle between leprosy skin and non-leprosy skin.

Sample		Leprosy patients (Skin)	Non-leprosy (Skin)	Chi-square *p*-value
		***n* = 30 (%)**	***n* = 15 (%)**	
Stained cells in eccrine glands % (Hes-1)	< 1%	10 (33.4%)	0	<0.0001
	1–25%	14 (46.6%)	0	
	25–75%	4 (13.4%)	0	
	> 75%	1 (3.3%)	14 (93.4%)	
	Not observed	1 (3.3%)	1 (6:6%)	
		***n* = 18 (%)**	***n* = 14 (%)**	
Stained cells in hair follicle % (Hes-1)	< 1%	7 (38.9%)	0	<0.0001
	1-25%	8 (44.4%)	0	
	25-75%	3 (11.1%)	0	
	> 75%	0	14 (100%)	

Differences in Cyclin D1 Expression in Nerve Fibers of Leprosy Patients and Non-leprosy Individuals

When determining the lack of overexpression of Notch components in dermal nerve fibers in leprosy patients, it was feasible that the neural damage model was not related to cyclin D1 expression (8). We therefore decided to evaluate the

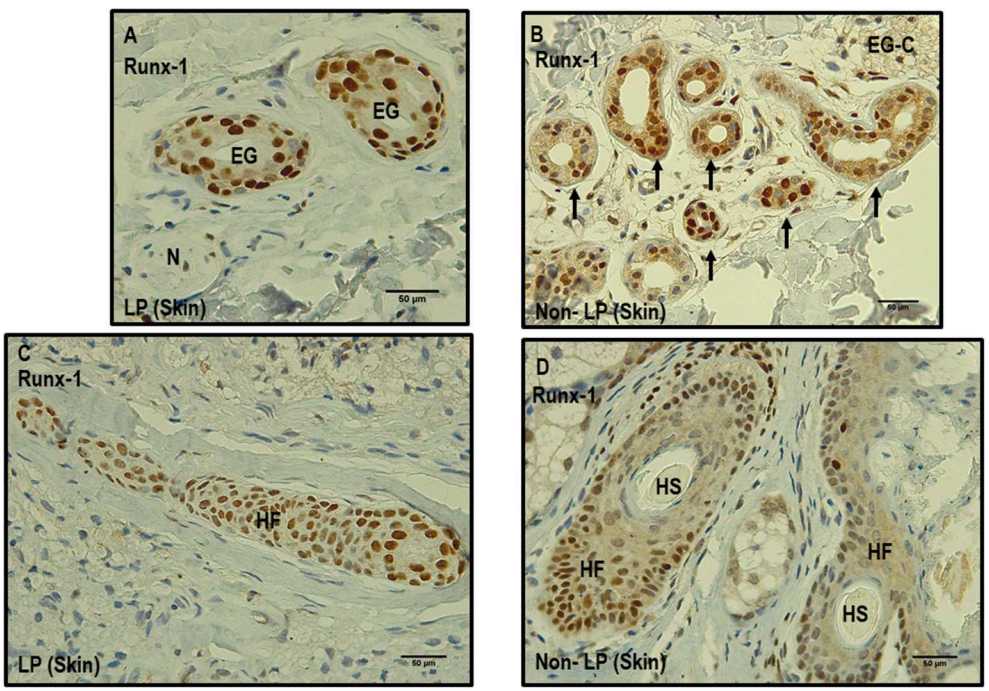

FIGURE 4 | Comparison between leprosy skin and non-leprosy skin using immunohistochemistry with Runx-1 in eccrine glands and hair follicles. **(A)** Two eccrine glands in leprosy sample (LP) positive for Runx-1; N = nerve. **(B)** Eccrine gland conglomerate (EG-C) in a non-leprosy sample (non-LP) positive for Runx-1. **(C)** Hair follicle (HF) in a leprosy sample (LP) positive for Runx-1 **(D)**. Two hair follicles and two hair shafts (HF) in a non-leprosy sample (non-LP) positive for Runx-1. All images have a 50-μm scale bar.

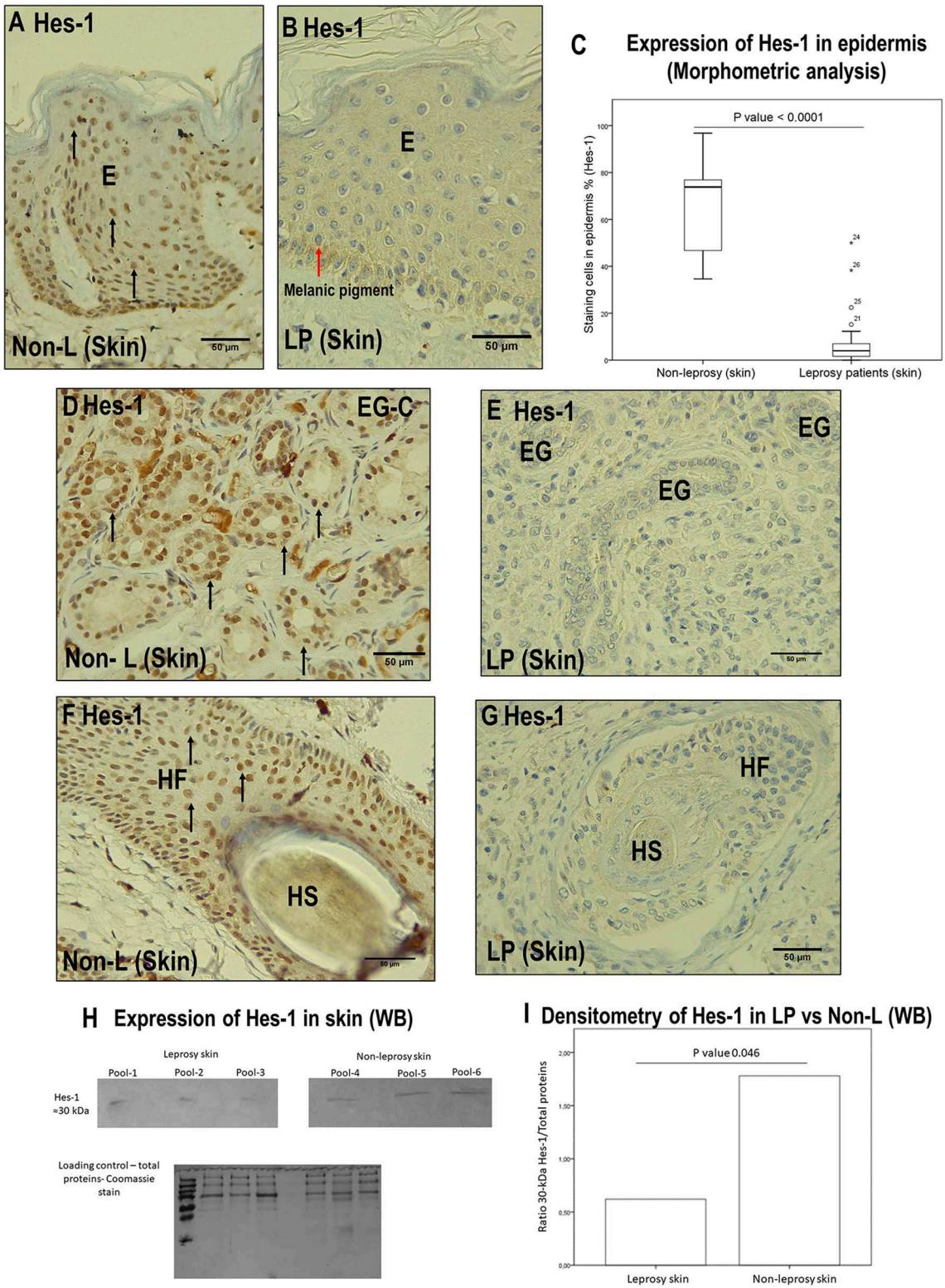

FIGURE 5 | Comparison between leprosy skin and non-leprosy skin using immunohistochemistry with Hes-1 in the epidermis, eccrine glands, and hair follicles. **(A)** Epidermis (E) of a non-leprosy sample (non-LP) positive for Hes-1 in the Malpighian layer. **(B)** Epidermis (E) of a leprosy sample (LP) negative for Hes-1. The red arrow shows melanic pigment. **(C)** Whisker box chart showing a lower expression of Hes-1 in the epidermis of leprosy patients vs. non-leprosy patients, $p < 0.0001$. **(D)** Conglomerate of eccrine glands (EG-C) in a non-leprosy sample (non-LP) positive for Hes-1. **(E)** Three eccrine glands (EG) in a leprosy sample (LP) negative for Hes-1.

(Continued)

FIGURE 5 | (F) Hair follicle (HF) and hair shaft (HS) in a non-leprosy sample (non-LP) positive for Hes-1. **(G)** Hair follicle (HF) and hair shaft (HS) in a leprosy sample (LP) negative for Hes-1. **(H)** Western blot showing the bands for Hes-1 in leprosy skin, non-leprosy skin, and loading control (total proteins stained with Coomassie). **(I)** Densitometry showing a lower expression of Hes-1 in leprosy patients vs. non-leprosy patients, $p < 0.05$. All images have a 50-μm scale bar.

TABLE 6 | Multivariate analysis of the histopathological and molecular variables that could explain the changes observed in the expression of Hes-1.

Variables	Epidermis		Eccrine glands		Hair follicle	
	Chi-square	*p*-value	Chi-square	*p*-value	Chi-square	*p*-value
Tissue inflammation, Atrophic changes, Dermal nerve damage, *Jagged-1, Notch-1*	**73.27**	**<0.0001**	**73.86**	**<0.0001**	**89**	**<0.0001**
Inflammation	**19.9**	**<0.0001**	**25.35**	**<0.0001**	**27.05**	**<0.0001**
Atrophic changes	7.8	0.05	0.425	0.98	4.83	0.305
Dermal nerve changes	10.8	0.095	5.4	0.66	10.86	0.209
Jagged-1	7.8	0.05	**14.18**	**0.007**	**9.8**	**0.044**
Notch-1	4.9	0.17	8.31	0.081	2.8	0.579

The bold values are showing statistical significance.

expression of cyclin D1 in the dermal nerves of a subsample of 10 leprosy and five non-leprosy samples.

With regard to the expression of cyclin D1 in the nerve fibers, 100% ($n = 10$) of leprosy samples showed no cyclin expression in the evaluated dermal nerves. Similarly, 100% ($n = 5$) of non-leprosy samples showed no expression of this cellular component in the nerves (**Figure 6**).

DISCUSSION

Differences in the Expression of Some Components of the Notch Signaling Pathway Between the Cutaneous Samples of Leprosy Patients and Non-leprosy Individuals

When evaluating the transcription of genes related to the Notch signaling pathway, we found significant changes in the expression of *Hes-1* and *Runx-1* in leprosy samples, in fact, *Hes-1* gene was found to be downregulated in the samples of leprosy patients This allows us to infer that *Hes-1* would not be related to the deterioration of the dermal nerve fiber caused by *M. leprae*, given that *Hes-1* overexpression is necessary to induce nerve damage (10).

On the other hand, the reduction of *Hes-1* in the cutaneous samples of these patients makes us think that *M. leprae* could directly induce trophic alterations in the skin. This finding is related to what was reported by Lin et al. (20), who demonstrated that the reduction of Hes-1 compromises differentiation and cell proliferation in the epidermis and hair follicle, together with the pluripotential capacity of stem cells in the skin.

In addition, the compromise of *Hes-1* could be explained through results from this research, such as the reduction in the expression of the *Jagged-1* ligand and even the *Notch-1* receptor in skin affected by *M. leprae*. Since *Jagged-1* ligand–*Notch-1* receptor interaction does not occur, the cleavage and translocation of the Notch intracytoplasmic domain (NICD) at the nuclear level would be impaired, thus blocking the expression of *Hes-1* (6, 21).

The results of the increased transcription of *Runx-1* in the cutaneous samples of leprosy patients allow us to consider two hypotheses in this respect. The first is that the overexpression of this transcription factor occurs in the dermal nerve fiber, which could lead to its deterioration (10). Our second hypothesis is that the increase in the transcription of *Runx-1* is a consequence of its canonical expression in immune cells such as macrophages (22, 23), cells that have a marked activity *in situ* against *M. leprae* in the skin of MB patients (24).

With respect to *Hey-1*, no significant changes in its expression were observed, although it has been related to the genes of the *Hes* family in the processes of proliferation and cellular differentiation at cutaneous and nervous levels (25, 26). It is possible that this finding is related to what was reported in murine keratinocytes by Blanpain et al. (27), who found that *Hey-1* expression is significantly reduced with respect to *Hes-1* from embryonic day 17, and this would be related to an increased activity of Hes-1 in cell proliferation processes at the epidermal level.

Among the genes evaluated, we also expected to find an increase in *Numb* transcription, which is a Notch inhibitory membrane protein with the ability to bind and prevent cleavage and nuclear translocation of the NICD, thus blocking the transcriptional machinery of this signaling pathway (28). Therefore, in the absence of a difference in *Numb* expression between leprosy and non-leprosy samples, we rule out that the changes in *Notch* expression found in the skin of these patients are mediated by changes in the expression of this inhibitory protein.

Based on these findings, we proposed that changes in the expression of *Hes-1* in the skin of these patients could compromise the proliferation, differentiation, and immune response of dermal cells against this microorganism, and at this point in the research, we did not rule out that *Runx-1* expression was occurring in the dermal nerve fiber. It is for this reason that the immunohistochemical evaluation of Hes-1 and Runx-1 became necessary so as to establish the dermal structures in which the changes in the expression of these genes were taking place.

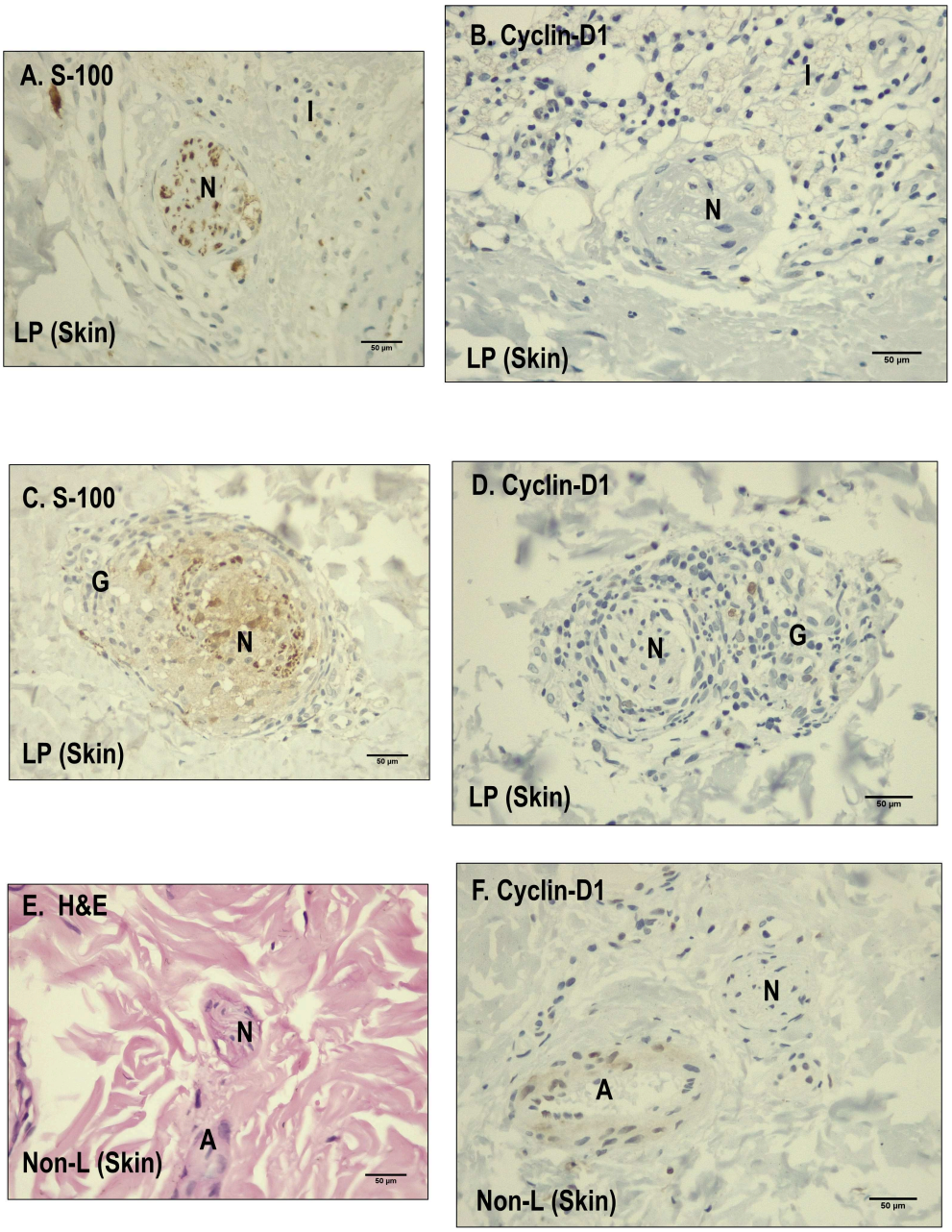

FIGURE 6 | Comparison between leprosy skin and non-leprosy skin using immunohistochemistry with Cyclin D1 in dermal nerves. **(A)** Dermal nerve (N) stained with S-100 in a leprosy sample. **(B)** Dermal nerve (N) surrounded by inflammatory cells in a leprosy sample (LP) negative for Cyclin D1. **(C)** Dermal nerve surrounded by a granuloma in a leprosy sample (LP) stained with S-100. **(D)** Dermal nerve surrounded by a granuloma in a leprosy sample (LP) negative for cyclin D1. **(E)** Dermal nerve (N) stained with hematoxylin and eosin (H&E) in a non-leprosy sample (non-LP); A, Artery. **(F)** Dermal nerve (N) in a non-leprosy sample (non-LP) negative for cyclin-D1; A, artery.

Cutaneous Structures in Which There Are Differences in the Expression of Runx-1 in Leprosy Patients and Non-leprosy Individuals

IHC tests corroborated that the increase of Runx-1 in leprosy patients is linked to the presence of macrophages marked with CD68 in the inflammatory infiltrate. This finding would be related to the fact that Runx-1 is key to the maintenance and survival of these cells by inhibiting pro-apoptotic molecules such as Fas receptor and BH3-only protein Bim (22). On the other hand, the non-expression of Runx-1 in the dermal nerve fiber demonstrates that it is likely that this transcription factor is not a trigger for demyelination and nerve fiber damage.

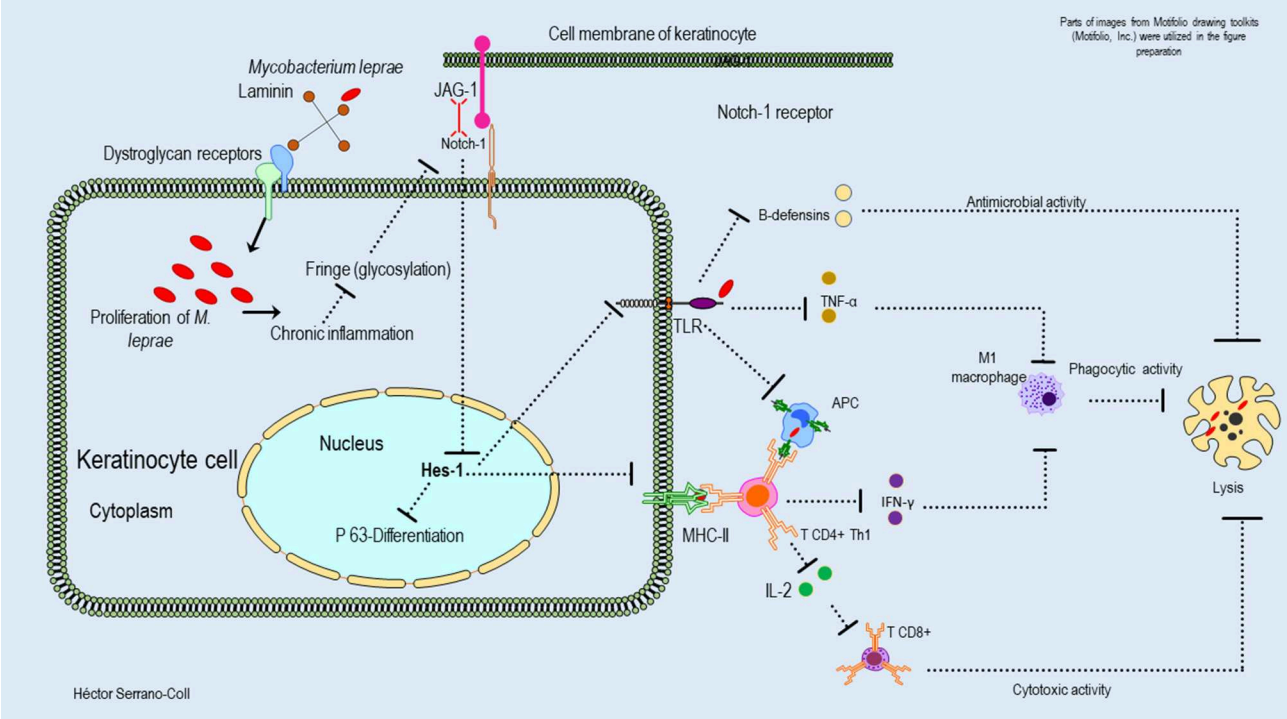

FIGURE 7 | Hypothetical model of the possible mechanisms used by *M. leprae* to modulate the expression of the Notch signaling pathway in the epidermis and their consequences. This figure shows how *M. leprae* could be related to the basement lamina (laminin receptor–dystroglycan complex) of the keratinocytes. The proliferation of *M. leprae* inside of these cells could induce a chronic inflammatory process that would reduce the glycosylation of the Notch receptor and its interaction with the ligand Jagged-1. This fact could induce aberrant signaling and reduced expression of Hes-1, affecting the activation of the protein P-63, the differentiation of the keratinocytes, and the innate and adaptative immune response against *M. leprae*.

As for the expression of Runx-1 at the level of the eccrine glands and hair follicles, to date, it is known that Runx-1 is a transcription factor that has been related to promoting stem cell activity at the level of the hair follicle (29, 30). In addition, this work could establish that Runx-1 is also expressed in the epithelium of the eccrine glands and that its action could be related to promoting the processes of stem cell proliferation and differentiation located on this cutaneous structure.

These findings exclude that the transcription factor Runx-1 is a trigger in the damage of the dermal nerve fiber or is involved with tissue changes in the skin of leprosy patients.

Cutaneous Structures in Which There Are Differences in the Expression of Hes-1 in Leprosy Patients and Non-leprosy Individuals

The IHC evaluation allowed us to establish that the reduction in the expression of Hes-1 in the skin of leprosy patients is limited to three skin structures: epidermis, eccrine glands, and hair follicles. This is in comparison with non-leprosy individuals, in whom Hes-1 was expressed constitutively in these structures, according to Cleaton et al. (31).

The reduced expression of Hes-1 in the epidermis of leprosy patients at the keratinocyte level would have a direct effect on the mechanisms of proliferation and differentiation in this cell (25). It is known that Hes-1 can directly induce the proliferation of keratinocytes by promoting the progression of the cell cycle at the level of the basal layer of the epidermis (6). It also facilitates the expression of the protein P-63, which is a key regulator in the development and differentiation of this cell-type, by inducing the expression of cytokeratins such as K1 and K10 (32, 33). Therefore, trophic changes in the epidermis of leprosy patients could be explained by *M. leprae*-induced neural damage and changes in Hes-1 expression. In addition, it is likely that changes in the expression of Hes-1 and Notch ligands and receptors will compromise the expression of Toll-like receptors (TLR) and major histocompatibility complex class II (MHC-II) molecules (34). Therefore, this cell de-differentiated would alter the innate immune response, the antimicrobial activity, the antigenic presentation, and the differentiation of CD4+ LT toward a Th1 and Th17 pattern, which are key in the activation of cells with cytotoxic and phagocytic activity against *M. leprae*. **Figure 7** shows a hypothetical model of the possible mechanism used to *M. leprae* to modulate the Notch signaling pathway in the epidermis.

Changes in the expression of Hes-1 in the skin annexes (eccrine glands and hair follicles) of leprosy patients could compromise the pluripotential activity of stem cells located in the eccrine glands and in the promontory of the hair follicle

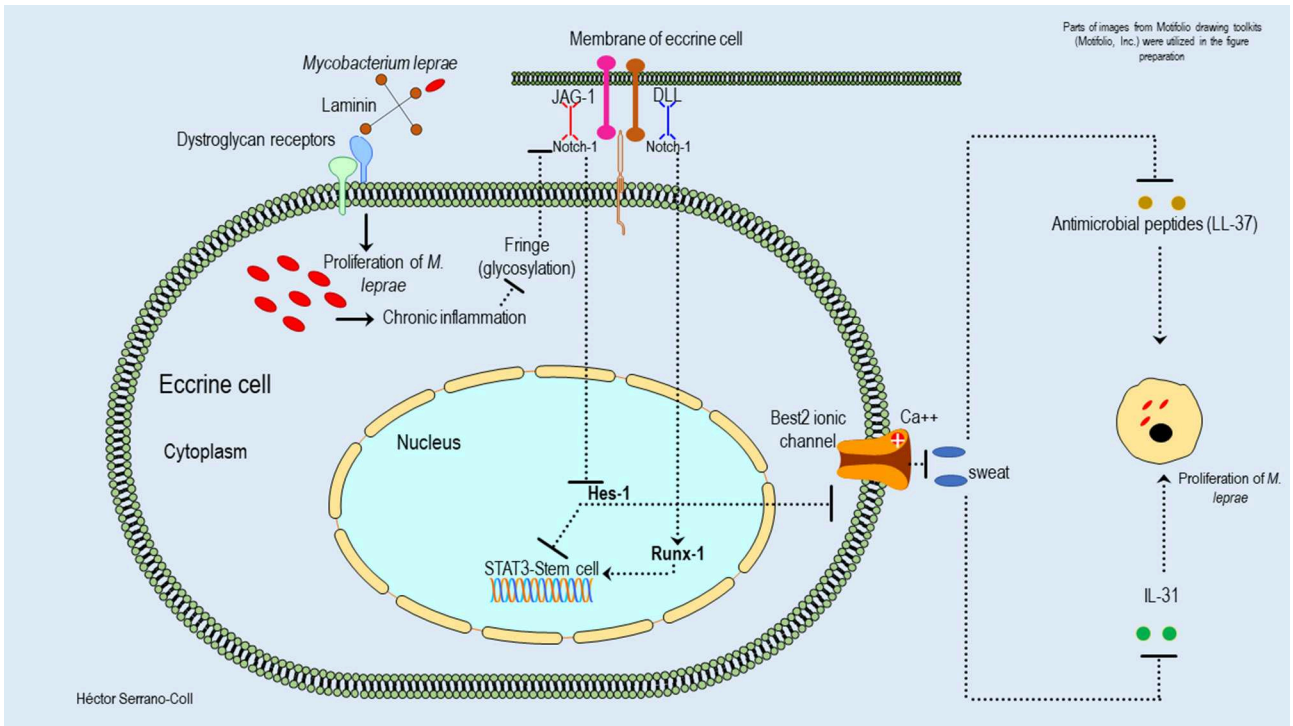

FIGURE 8 | Hypothetical model of the possible mechanisms used by *M. leprae* to modulate the expression of the Notch signaling pathway in eccrine cells and their consequences. This figure shows how *M. leprae* could reduce the expression of Hes-1 in the eccrine cells. This would block the activation of STAT3 and the function of the stem cells located in this cutaneous annex. This effect could be partially mitigated by Runx-1. In addition, the reduction of the expression of Hes-1 could be associated with the reduction of sweat secretion, probably because the inhibition of the expression of the Best-2 channels, with a consequent reduction of secretion of antimicrobial peptides (cathelicidin LL-37) and cytokines (IL-31), could promote the proliferation of *M. leprae* and the interaction host-pathogen.

(35, 36), considering that Hes-1 is key in the activation of STAT3 (Signal Transducer and Activator of Transcription 3), which is a transcription factor involved in the maintenance and differentiation processes of stem cells (37). **Figures 8**, **9** show a hypothetical model of the possible mechanism used to *M. leprae* to modulate the Notch signaling pathway in eccrine glands and hair follicles, respectively.

These findings allow us to establish, that alterations in the processes of reepithelialization and scarring in the skin lesions of leprosy patients could be related both to tissue compromise generated by leprosy neuropathy (38) and to changes in Hes-1 expression. In addition, we consider that in the future, modulation of this transcription factor could be a therapeutic alternative in the management of leprosy ulcers.

In the eccrine glands, it is also important to mention that the reduction of Hes-1 would compromise the secretory function of the luminal cells, which is mediated by ionic channels dependent on calcium, called Bestrophin-2 (Best2) (39). These channels have been extensively studied at the intestinal level, showing that the secretory activity of Best2 is dependent on the Notch signaling pathway and specifically on the expression of Hes-1 (40). Therefore, changes in its expression would reduce sweating, its antimicrobial activity (cathelicidin LL-37), and the expression of cytokines such as IL-31—key in the activation of keratinocytes (39)—in the cutaneous areas affected by *M. leprae*, which would facilitate the proliferation of this mycobacterium (**Figure 8**).

Another relevant finding of this research is that we did not find expression of Hes-1 in the nerve fibers of leprosy patients, which makes it unlikely that Hes-1 is involved in the deterioration of dermal nerve fibers (10).

Histopathological and Gene Changes That Might Explain the Reduction in Hes-1 Expression in the Skin of Leprosy Patients

The main histopathological finding of this research associated with the reduction in the expression of Hes-1 in the skin of leprosy patients is the inflammation found at the level of the dermis, skin annexes, and subcutaneous cellular tissue. This could be related to the findings of Derada et al. (41), who observed that inflammatory processes at the cellular level reduce the expression of a glucosyltransferase such as "Fringe," which has the function of adding residues of O-fucose to N-acetylglucosamine repetitions, located in the extracellular domain of the Notch receptor (NECD). The interaction of the ligand Jagged-1-receptor Notch-1, which activates this signaling pathway, depends on this glycosylation (42). Therefore, we propose that inflammation induced by *M. leprae* in the skin could cause alterations in the glycosylation of NECD, causing

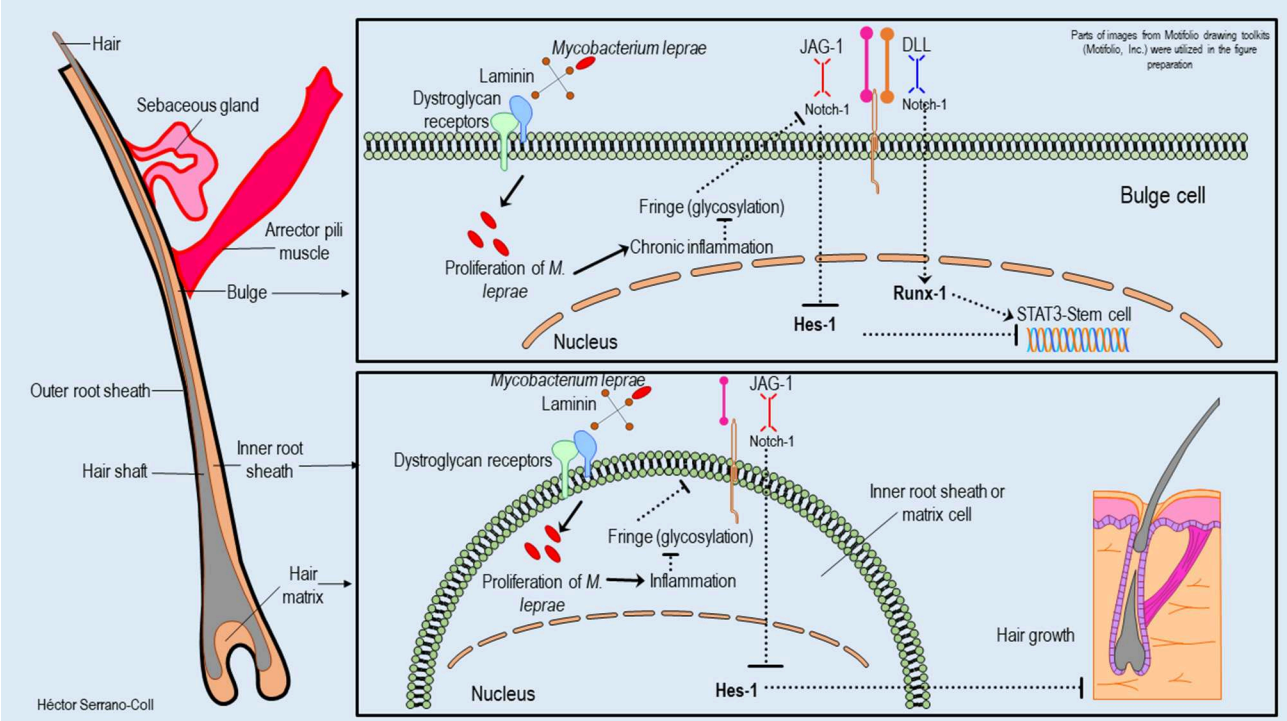

FIGURE 9 | Hypothetical model of the possible mechanisms used by *M. leprae* to modulate the expression of the Notch signaling pathway in the hair follicle and their consequences. This figure shows how *M. leprae* could reduce the expression of Hes-1 in the hair follicle. This fact would block the activation of STAT3 and the function of stem cells located in the bulge, which is partially mitigated for the expression of Runx-1. In addition, the reduction of the Hes-1 expression could induce the loss of hair follicles in the skin of leprosy patients.

aberrant signaling of Notch and changes in the expression of Hes-1 (**Figure 7**).

In the epidermis, eccrine glands, and hair follicle another finding is that the expression of Hes-1 could be related to changes in the gene expression of the *Jagged-1* ligand since the reduced expression of this cellular component would limit the ligand-receptor interaction and would reduce the expression of Hes-1 (6, 21), as discussed previously.

On the other hand, based on the inflammatory mechanism explaining the reduction of Hes-1, we ask the question: why was the transcriptional factor Runx-1 not altered in leprosy patients, considering that some Notch transcription factors are affected in inflammatory environments? The answer we suggest is that Runx-1 can be expressed through two pathways, one that is given by the interaction of Jagged ligands with the Notch-1 receptor and a second that is mediated by the union of Delta-type ligands with this receptor (43); it has been demonstrated that this interaction (Delta-Notch) is not involved in inflammatory processes (41), which confirms our findings.

Cyclin D1 Expression in Dermal Nerves of Leprosy Patients and Non-leprosy Individuals

Since there is no evidence of cyclin D1 expression in the dermal nerve fibers of leprosy patients, we rule out the possibility that neural damage in these patients has related to cyclin D1 expression or to the transcription factors of the Notch signaling pathway that were evaluated. It is probable that in these patients, dermal nerve fiber damage is linked to the reduction of transcription factors Oct-6 and Sox-10, which are positive regulators of the genes in charge of myelin maintenance in the nerve (unpublished data).

The absence of cyclin D1 in the dermal nerve fibers affected by *M. leprae* would be related to the lack of expression of transcription factors Hes-1 and Runx-1. Given that, if there is no cyclin D1 overexpression, it is unlikely that the expression of Numb—which is a Notch inhibitory protein—will be reduced, thus limiting cleavage and translocation at the nuclear level of the NICD, thus making the expression of Notch-associated transcription factors, which behave as a myelin repressor in the nerve, unfeasible (10).

CONCLUSIONS AND FUTURE PERSPECTIVE

The Notch signaling pathway is a cellular component that is not involved in the deterioration of the dermal nerve fiber but is related to some *M. leprae*-induced tissue changes in the skin of its host (epidermis, eccrine glands, and hair follicles). Such changes would be linked to a reduced expression of the transcription

factor Hes-1, which then alters the processes of proliferation and differentiation in keratinocytes, eccrine luminal cells, and cutaneous stem cells, which would allow *M. leprae* to survive and proliferate in this tissue.

Around this infection and its relationship with the Notch signaling pathway, we still need to resolve some questions, such as:

1. What other components of the Notch signaling pathway could be involved in *M. leprae*-induced tissue and cell damage?
2. Could Runx-1 expression be a compensatory mechanism in the skin of leprosy patients, useful for coping with the tissue changes induced by the reduction in Hes-1?
3. How can we intervene in the modulation of Notch activity and restore Hes-1 expression in the skin of leprosy patients?
4. Could Hes-1 be used as an auxiliary marker in the diagnosis of leprosy?

These questions indicate that there is still a long way to go in understanding the cellular mechanisms proposed here. In addition, it is likely that from these findings or others related to changes in cell signaling induced by *M. leprae*, new and better tools will be developed in the future to facilitate the early detection of leprosy and allow us to take a step forward in the elimination of this disease.

LIMITATIONS

This research was focused on MB patients because the aim of this study was to understand a mechanism of direct tissue damage related to the ability of *M. leprae* to modulate a signaling pathway such as Notch. Therefore, in the short term, it is necessary to study this mechanism in PB patients and to show whether changes of the Notch signaling pathway are present in this clinical spectrum.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This research was conducted in accordance with the international ethical standards given by the World Health Organization and the Pan American Health Organization, supported by the Declaration of Helsinki, promulgated in 1964, and the statutes given at the national level by resolution number 008430 of 1993 of the Ministry of Health of Colombia, which regulates health studies. In addition, it was endorsed by the institutional ethics committee for human research of CES University (act No 101 of March 3, 2017), and respective endorsement was obtained from the ethics committee or the legal representative of the institutions that participated in this project.

AUTHOR CONTRIBUTIONS

HS-C and NC-C designed the study, standardized the qRT-PCR and WB assays, and wrote the manuscript. Participants in this study were evaluated by HS-C. HS-C, JO, and LS-P standardized the IHC tests. JO interpreted the study's histopathological and immunohistochemical findings. HS-C performed the data analysis. JO and LS-P conducted a critical review. This research was directed by NC-C. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00368/full#supplementary-material

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Phenotypical Characterization of Spleen Remodeling in Murine Experimental Visceral Leishmaniasis

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Background: Visceral leishmaniasis (VL) is caused by *Leishmania infantum* or *L. donovani* infection. One of the main problems related to this disease is the emergence of severe clinical forms with a lethality of 5–20%, even while under specific treatment. In humans and other species susceptible to fatal VL, such as dogs and hamsters, the disruption of splenic white pulp (WP) is accompanied by disease progression. Control of VL progression is seen in BALB/c mice, as evidenced by a mild clinical presentation and controlled parasite replication in the liver and spleen. In this study, we investigated the features involved in the morphological remodeling of splenic compartments associated with the control of VL progression to death.

Methods: We evaluated cohorts of BALB/c mice after 30, 60, and 90 days of infection by *L. infantum*. Spleen morphology, cell population subsets and cytokine production were studied in the spleen using flow- and histo-cytometry.

Results: Intraperitoneal infection with 10^8 promastigotes of *L. infantum* led to progressive increases in spleen size at 60 and 90 days after infection. Splenomegaly was the only clinical sign of disease observed. At 30 days after infection, hyperplasia in the WP and decreased numbers of plasmacytoid dendritic cells were observed. The WP hyperplasia subsided at 60 days post-infection. However, the splenomegaly remained in association with increased numbers of macrophages, B and T lymphocytes and plasma cells. An increased number of lymphoid tissue inducer (LTi) cells was observed; these were distributed around the periarteriolar lymphoid sheath in control mice and scattered throughout the red pulp in the *Leishmania*-infected mice. After 90 days of infection, increased IL-6 and IFN-γ production was seen in the spleen, as well as higher frequencies of follicular and plasmacytoid dendritic cells.

Conclusion: The data presented herein emphasizes the potential role of spleen remodeling in the control of severe forms of VL and highlights features potentially involved in this process.

Keywords: visceral leishmaniasis, white pulp remodeling, spleen disorganization, lymphoid tissue inducer cells, spleen pathology

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INTRODUCTION

Visceral leishmaniasis (VL) is endemic in Central and South America, Asia, parts of Africa and the Mediterranean basin, with an estimated burden of 2.1 million DALY (disability adjusted life years) (1). It is a severe systemic parasitic disease caused by the protozoan *Leishmania donovani*, which affects humans, and *Leishmania infantum*, which affects humans and dogs. The current therapeutic approach with administration of glucantime, pentamidine or amphotericin is effective in most cases (2). However, VL is lethal in humans and dogs, even those under treatment. In Brazil, a ∼37,209 individuals were affected by VL between 2001 and 2011, with a lethality rate of 6.8% (2,549 deaths) (3).

The main clinical signs of VL are weight loss, increased size of the liver and spleen, anemia, low platelet and neutrophil counts and increased susceptibility to bleeding and coinfections, leading to death (4, 5). The disease results from an inability of macrophages to kill the parasite (6). A complex signaling network of molecules produced by macrophages and T and B lymphocytes, with pro- and anti-inflammatory roles, produces an inflammatory status that is responsible for the many clinical manifestations of the disease without achieving control of parasite growth (5, 7). The bone marrow, liver and spleen are the most affected internal organs (8).

The spleen is a large lymphoid organ responsible for many physiological functions such as hemocatheresis and it is also responsible for immune surveillance against blood circulating pathogens (9). The spleen is organized into two main areas: white pulp (WP) and red pulp (RP). It is affected by *Leishmania* infection in all cases and during the entire course of the disease (10). Although the spleen compartments contain the crucial elements to effectively respond to *Leishmania* infection, in severe cases of disease, the spleen undergoes sequential changes of WP hyperplasia, atrophy and disruption (11). Spleen enlargement leads to hypersplenism syndrome with increased leukocyte and platelet retention and destruction of blood cells (12, 13).

In the late stages of severe VL, the WP is disrupted, germinal centers and mantle zones disappear, and lymphoid follicles are barely defined (14, 15). These changes are associated with decreased number of B lymphocytes, increased apoptosis of T lymphocytes, loss of follicular dendritic cells (FDCs), high parasite burden and change in the cytokine expression pattern (16–18). Loss of FDCs impairs production of CXCL13, a chemokine involved in B cell recruitment into the lymphoid follicles (19). Consequently, the B cells migrate to the RP where they differentiate into plasma cells (15), where overexpression of BAFF, APRIL, and CXCL12 contribute to an extended survival time of these cells (20). Progressive splenomegaly and remodeling of the splenic compartments are observed in experimental murine VL. Although extensive WP disruption was only observed after 60 days of infection, redistribution of marginal zone macrophages as well as RP vascular network remodeling were observed at 28 days post-infection (dpi) (11, 21, 22). The progressive lymphoid follicle depletion in murine VL was dependent on the initial inoculum size and the infection time (11, 23). Altogether, these alterations may interfere with memory T cell and B cell responses and contribute to an exacerbated and ineffective humoral immune response. The sequential cellular and molecular events leading to spleen compartment disorganization in VL still need to be elucidated. The fact that spleen disorganization is associated with more severe, sometimes, terminal disease, suggests that it plays a role in the progression of VL to a stage of no-response to current therapeutic approaches.

Lymphoid tissue inducer (LTi) cells are type 3 innate lymphoid cells (ILC3) characterized by expressing CCR6 with variable expression of CD4 (24, 25). In mice, these cells can be identified by expressing CD4 and not expressing lineage markers (e.g., CD3, B220, CD11c) (26). LTi cells interact with immune and stromal cells thereby promoting lymphoid tissue organogenesis such as lymph nodes and Peyer's patches (27–29). Although these cells are not critical for splenic WP development, they may provide early lymphotoxin signals in T cell areas and continue to play a role in WP organization in adult life (30, 31). For instance, LTi cells have been reported to participate in WP repair after injury caused by choriomeningitis virus infection (32). However, upon infection of mice with *L. donovani,* LTi cells appeared not to be crucial for splenic lymphoid tissue restoration induced by Sunitinib maleate (a tyrosine kinase inhibitor) (33).

In this study, we attempted to induce an extensive morphological disruption of the splenic WP in BALB/c mice upon prolonged infection with *L. infantum*, similar to that reported by Veress et al. (11). Our aim was to use this model to address the phenotypic cell changes leading to the profound disruption of the WP associated to severe chronic forms of VL. The data presented show that even heavily infected mice with a high parasite inoculum do not develop similar extensive spleen disorganization as seen in hamsters, dogs and humans. Furthermore, we observed changes in LTi cells distribution that may support a role played by these cells in the reorganization of splenic WP in the murine experimental model of infection with *L. infantum*.

MATERIALS AND METHODS

Mice

BALB/c mice were obtained from the colony of IGM-FIOCRUZ. The animals were allocated into homogeneous experimental groups on the basis of sex, weight and age. They were kept in the experimental areas with food and water available *ad libitum* and under a controlled physiological regime of temperature and periods of light and dark.

Parasites and Injection

L. infantum promastigotes (strain MHOM/BR2000/Merivaldo2) were maintained in passage in Golden Syrian hamsters and cultured *in vitro* until the stationary phase in complete Schneider medium (Schneider + 20% fetal bovine serum [FBS], Gibco, USA) in a B.O.D. incubator at 24°C. Mice

were injected intraperitoneally (i.p.) at 6–8 weeks of age with either saline solution (control) or a parasite suspension of 10^7 (first experiment) or 10^8 (second experiment) promastigotes. Euthanasia was performed by overdose of anesthetics (10 mg cetamin + 1 mg xylazine/mL) at 30, 60, and 90 days post injection (dpi). The spleen was removed and divided into four fragments to perform flow cytometry, histology, histo-cytometry and qPCR. Clinical examination was performed according alterations of weight and hair loss, dehydration, skin lesions and splenomegaly. Evidence of infection was obtained by isolation and culture of parasites from the spleen (34). The parasite burden of the spleen was determined using qPCR as previously described (35). Anti-*Leishmania* antibody activity was detected by enzyme-linked immunosorbent assay (ELISA) in diluted (1:200) mouse serum as previously described (36).

Isolation of Cells

For cell and cytokine flow cytometry analysis, the larger fragment of the spleen was weighed, then macerated in a cell strainer at 70 μm (BD Falcon) coupled to a 50 mL conical tube filled with phosphate-buffered saline (PBS). The cell suspension was centrifuged at 1,600 rpm/5 min/4°C. The cell-containing pellet was incubated in 2 mL of Red Blood Cells Lysis Buffer (BD Biosciences) for 2 min/37°C then centrifugated at 1,400 rpm/5 min/4°C. The cells were resuspended in 1 mL PBS and then the cell viability was assessed by trypan blue staining.

Immunophenotyping of Leukocytes Using Flow Cytometry

Cell suspensions were incubated in FBS buffer with 5% mouse serum to block non-specific reactions. The cells were distributed in 100 μl of the suspension/well at a concentration of 2×10^6 cells/mL. The following antibodies were used, all from BD Biosciences: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (17A2) and anti-CD11b (M1/70); phycoerythrin (PE)-conjugated anti-CD4 (GK1.5) and anti-B220/CD45R (30-F11); PE-Cy5-conjugated anti-F4/80 (BM8); Alexa Fluor (AF) 700-conjugated anti-CD19 (1D3); brilliant violet (BV) 421-conjugated anti-CD11c (N418) and anti-CD138 (281-2); BV 605-conjugated anti-MHC-II (I-A/I-E, M5/114.15.2); and brilliant blue (BB) 515-conjugated anti-CD23 (B3B4) and anti-CD93 (AA4.1) (**Supplementary Table 1**). Cells were incubated for 20 min with antibodies for the markers of interest and their respective control isotypes. Subsequently, cells were washed twice with PBS buffer. Cells were acquired using an LSRFortessa flow cytometer (BD Biosciences, USA). Gating strategy and data analysis were performed using FlowJo software (Tree Star Inc., California, USA).

Splenocytes Stimulation Assay and Cytokine Mensuration by Flow Cytometry

Cell suspensions were transferred into 96-well plates in duplicate at 5×10^5 cells. The plate was centrifuged at 2,000 g/10 s/4°C and the pellet was incubated with 100 μl of 50 μg/mL soluble *L. infantum* antigen (SLA) or 35 μl/mL concanavalin A (ConA) in Roswell Park Memorial Institute medium (RPMI) + 10% FBS. The plate was placed in a CO_2 incubator at 37°C for 48 h. Then, it was centrifuged at 2,000 g/10 s/25°C and the supernatant was collected. A CBA Mouse Inflammation Kit Th1/Th2 (BD Biosciences) was used for measurement of IL-6, IL-10, MCP-1, IFN, TNF, and IL-12p70 in the supernatant of the pre-stimulated cells. Beads were acquired using an LSRFortessa cytometer (BD Biosciences) and analysis was performed using FlowJo software (Tree Star Inc., California, USA).

Immunostaining and Image Acquisition

Spleen fragments were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Japan Co., Ltd.) and frozen in the vapor of liquid nitrogen. Cryostat sections 4 μm thick were cut and placed onto negatively charged glass slides. The sections were air-dried and fixed in acetone for 10 min. Tissues were blocked for non-specific binding using 10% normal mouse serum for 15 min. Sections were incubated with specific antibodies (**Supplementary Table 1**) diluted in PBS + 2% newborn calf serum (NCBS) for the stromal cells panel and LTi cells panel. Sections were stained with fluorochrome-conjugated antibodies for 30 min, washed twice with PBS and finalized with mounting medium. For indirect antibody binding, sections were incubated with primary antibodies for 45 min, washed twice with PBS, and then incubated with secondary antibody for 30 min. The slides were washed in PBS and finalized with mounting medium. Negative controls were added per slide and treated with unconjugated antibodies and/or incubation buffer only. A Leica SP8 confocal microscope was used for image acquisition using LASX software (Leica Microsystems). Nine adjacent images were acquired in × 400 magnification into a mosaic to observe a larger area.

Immunostaining of Stromal Cells

Stromal cells were immunostained using the following antibodies: anti-ER-TR9 (mAB derived from hybridoma cell culture supernatant) primary mouse antibody and AF 647-conjugated secondary antibody (Invitrogen); primary anti-mouse gp38 (anti-podoplanin 8.1.1, Developmental Studies Hybridoma Bank – DSHB –at University of Iowa, Iowa City, IA) antibody and AF 594-conjugated secondary antibody (Invitrogen); direct-labeled AF 488-conjugated anti-B220/CD45R (affinity-purified from hybridoma cell culture supernatant); AF 555-conjugated anti-MAdCAM (MECA79, affinity-purified from hybridoma cell culture supernatant); and BV 510-conjugated CD35 (8C12, BD Biosciences) (**Supplementary Table 1**). Images were acquired in 1024 × 1024 resolution and absolute intensity per area represented by each marker was measured by surface detailing by automatic software selection (Imaris, Oxford Instruments).

Histo-cytometry of Lymphoid Tissue Inducer Cells

The phenotype of the LTi subsets was defined using a primary mouse antibody anti-IL-7Rα (A7R34, affinity-purified from hybridoma cell culture supernatant) and an AF 594-conjugated secondary antibody (Invitrogen) and/or AF 488-conjugated CD4 (GK1.5, affinity-purified from hybridoma cell culture

supernatant). Cell lineages were immunostained with APC-conjugated CD11b (M1.70) and CD11c (N418) from eBioscience, and AF 647-conjugated B220 and CD3 (affinity-purified from hybridoma cell culture supernatant), detected by the same filter (Cy5) and emitted in the same range (660–665) as the signal of non-interest. The slides were washed in PBS, incubated with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) nuclear dye and finalized with mounting medium. Images were acquired in 2,048 × 2,048 resolution and an additional step of deconvolution of the image was performed using Huygens software (Scientific Volume Imaging). Strategy for image analysis was performed using Imaris software. Minimal fluorescence intensity was defined as the threshold for gating of co-localization and creation of a channel double positive for IL-7Rα and CD4. An arithmetic tool was used to subtract the fluorescence signal in two ways: (1) Lineage (CD11b/CD11c/CD3/B220) and CD4, to characterize the $CD4^{-}$LTi subset (lineage$^{-}CD4^{-}$IL-7Rα^{+}); and (2) Lineage (CD11b/CD11c/CD3/B220), to characterize the $CD4^{+}$LTi subset (lineage$^{-}CD4^{+}$IL-7Rα^{+}). The number of cells was manually assessed using a counting tool. Red dots were added over the $CD4^{-}$IL-7Rα^{+}LTi cells and green dots were added over the $CD4^{+}$IL-7Rα^{+}LTi cells to facilitate observation of the images. Dispersion of the $CD4^{+}$IL-7Rα^{+} LTi cells was estimated by drawing the closest distance between these cells and the boundaries of the WP, morphometrically assessed using Image-Pro Plus version 6.0 software (MediaCybernetics, United States).

Histopathology

Spleen fragments were fixed for 24–48 h in alcoholic acid formalin solution at room temperature. Spleen fragments were sliced to 3–4 mm thick, placed in histological processing cassettes and embedded in paraffin, then sectioned to 3–4 μm thick for staining with hematoxylin and eosin (H&E). The animal tissues were examined without prior knowledge of the groups, with the guidance of two pathologists (WLCdS and LARF). The intensity of inflammatory infiltrates, parasitism, fibrosis and cell degeneration were estimated. The spleen was further examined as described previously by Hermida et al. (37). Briefly, the degree of architectural organization of the spleen WP was classified as follows: well-organized when distinguishing the periarteriolar sheath from the lymphoid follicle, germinal center, mantle zone and marginal zone; slightly disorganized, which presents with atrophic or hyperplastic changes leading to a loss of definition of some WP regions, with their poorly individualized and distinct regions; and extensively disorganized, when the follicular structure is rarely distinct from the RP and T cell area (15, 37).

Expression and Analysis of the Results

Numerical values are presented in tables or graphs representing absolute numbers, means, medians or percentages or fold changes relative to the control estimates as indicated. Comparisons of medians between control and infected groups were performed using Mann-Whitney test. For comparisons involving more than two groups ANOVA or Kruskal-Wallis tests was used and when recommended followed by Tukey's multiple comparison test. Time variations between groups were analyzed using ANOVA and Tukey's multiple comparison test. The threshold for statistical significance was set at a $p < 0.05$.

RESULTS

General Characteristics of the Animals

Both experiments with inoculum of 10^7 (presented as supplementary data) or 10^8 presented similar results. However, the changes between infected and control groups were more expressive in the animals infected with 10^8 promastigotes. The main characteristics of the mice infected with 10^8 promastigotes are summarized in **Table 1**. As assessed by spleen culture and qPCR, evidence of infection by *L. infantum* was present in all infected mice, either infected with 10^7 (**Supplementary Table 2**) or 10^8 (**Table 1** and **Figure 1D**) promastigotes and absent in control mice. Anti-*Leishmania* serology was negative in all control mice. Positive anti-*Leishmania* antibody detection was observed in 4/7 infected mice at 30 dpi and in all infected mice at 60 and 90 dpi (**Table 1**). A significant increase in the optical density (O.D.) for anti-*Leishmania* antibody activity was observed in infected mice after 60 dpi (1.5 [1.1–1.8]) and 90 dpi (2.3 [2.1–2.3]) in comparison to control mice (0.1 [0.1–0.2], 0.2 [0.2–0.2], $p < 0.001$, 60 and 90 dpi, respectively) (**Figure 1D**). Parasite burden progressively

TABLE 1 | Evidence of infection and clinical evaluation of the mice infected with 10^8 promastigotes of *L. infantum*.

Parameters	30 dpi		60 dpi		90 dpi		*p*-value
	CT	INF	CT	INF	CT	INF	
N (%)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	6 (100)	
Evidence of infection by *L. infantum*							
Spleen culture	0	7 (100)	0	7 (100)	0	6 (100)	nt
Serology	0	4 (57)	0	7 (100)	0	6 (100)	nt
Clinical signs of VL							
Splenomegaly	0	0	0	7 (100)	0	6 (100)	nt
Spleen weight (g)[a]	0.1 ± 0.01	0.1 ± 0.004	0.1 ± 0.01	0.2 ± 0.05[b]	0.1 ± 0.01	0.5 ± 0.1[b]	<0.0001

CT, control group; INF, infected group; [a], spleen weight is expressed in average and standard deviation; [b], statistical difference between control and infected groups per time point, t-test; nt, not tested.

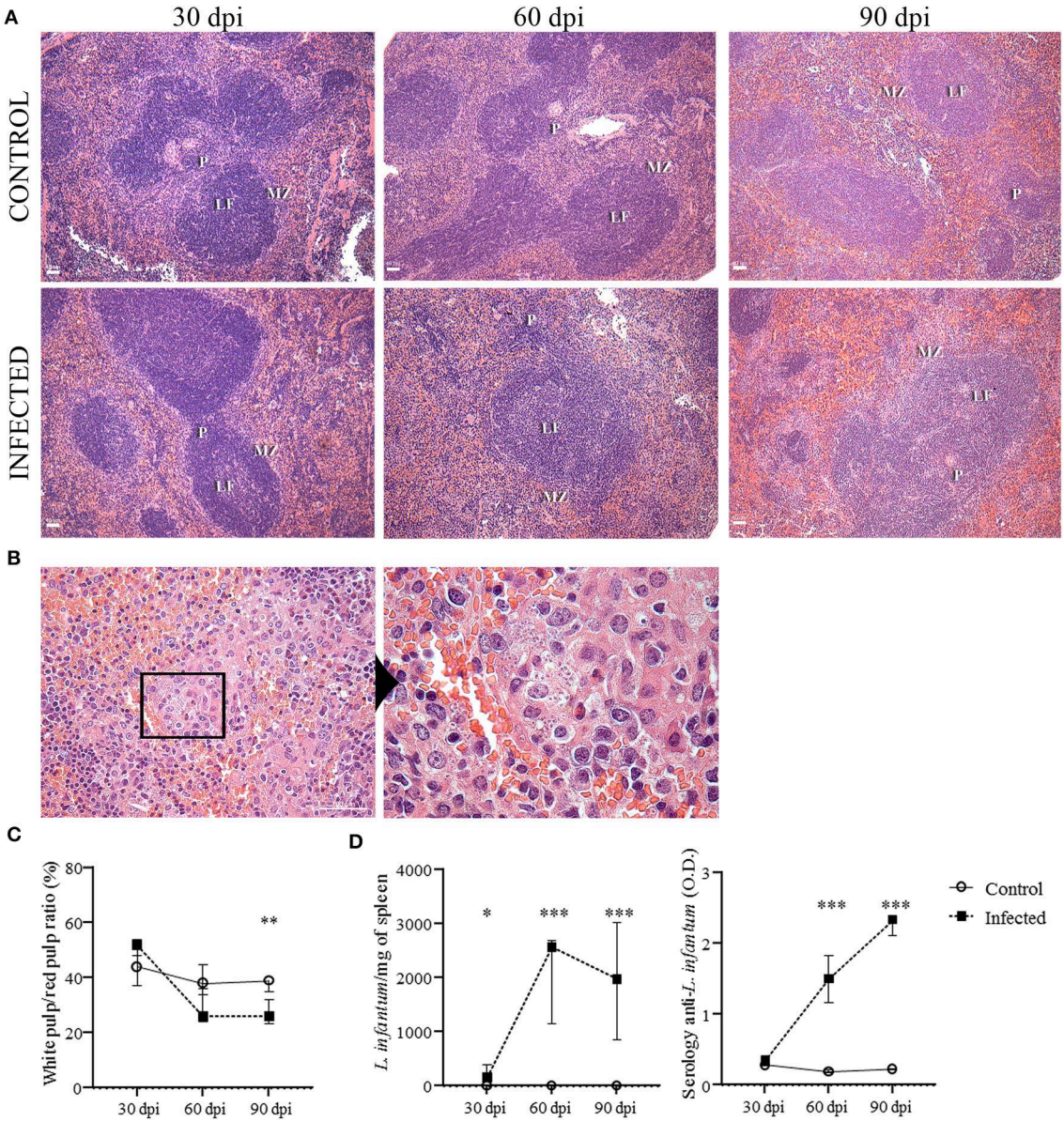

FIGURE 1 | Histological changes of the spleen and evidence of infection. **(A)** Spleens of uninfected (control) and *L. infantum*-infected mice at 30, 60- and 90-days post-injection (dpi) (bars = 40 μm). MZ = marginal zone; LF = lymphoid follicle; PALS = periarteriolar lymphoid sheath. **(B)** Granuloma in the spleen of an infected mouse at 90 dpi (bar = 100 μm) evidencing amastigotes of L. infantum. **(C)** Morphometric estimation of white pulp (WP) and red pulp (RP) area represented in percentage of WP/RP ratio. Difference between control and infected mice (90 dpi), $^{**}p = 0.006$, Mann-Whitney test. **(D)** *L. infantum* per milligram of spleen of infected and control mice after 30 ($^{*}p < 0.05$), 60 ($^{***}p < 0.001$) and 90 ($^{***}p < 0.01$) dpi (Kruskal-Wallis test). Optical density (O.D.) values for anti-*Leishmania* antibody activity in infected and control mice after 30, 60 ($^{***}p < 0.001$) and 90 ($^{***}p < 0.001$) dpi. The graphs represent the median and interquartile range (Kruskal-Wallis test).

increased from 30 dpi (155.1 *L. infantum*/mg of spleen [151.8–391.2]) to 60 dpi (2,561 [1,153–2,690], $p = 0.002$) and to 90 dpi (1,978 [857.1–3,007], $p = 0.01$), and was negative in all control mice. Splenomegaly was characterized by an increase in the size and weight of the spleen and was observed at 60 and 90 dpi in mice infected with 10^8 promastigotes (**Table 1**) but not in mice infected with 10^7 promastigotes (**Supplementary Table 2**).

Histological Evaluation of the Spleen

The splenic architecture was mainly preserved in all groups, although we observed mild disorganization of WP in 2/7 infected mice at 30 dpi and 1/6 infected mice at 90 dpi. This difference was not statistically significant (**Figure 1A**). There was a trend to a decrease in size of the WP area in the infected animals by 60 dpi. However, only at 90 dpi the area of the WP was significantly smaller in the infected compared to the control group (p = 0.006,

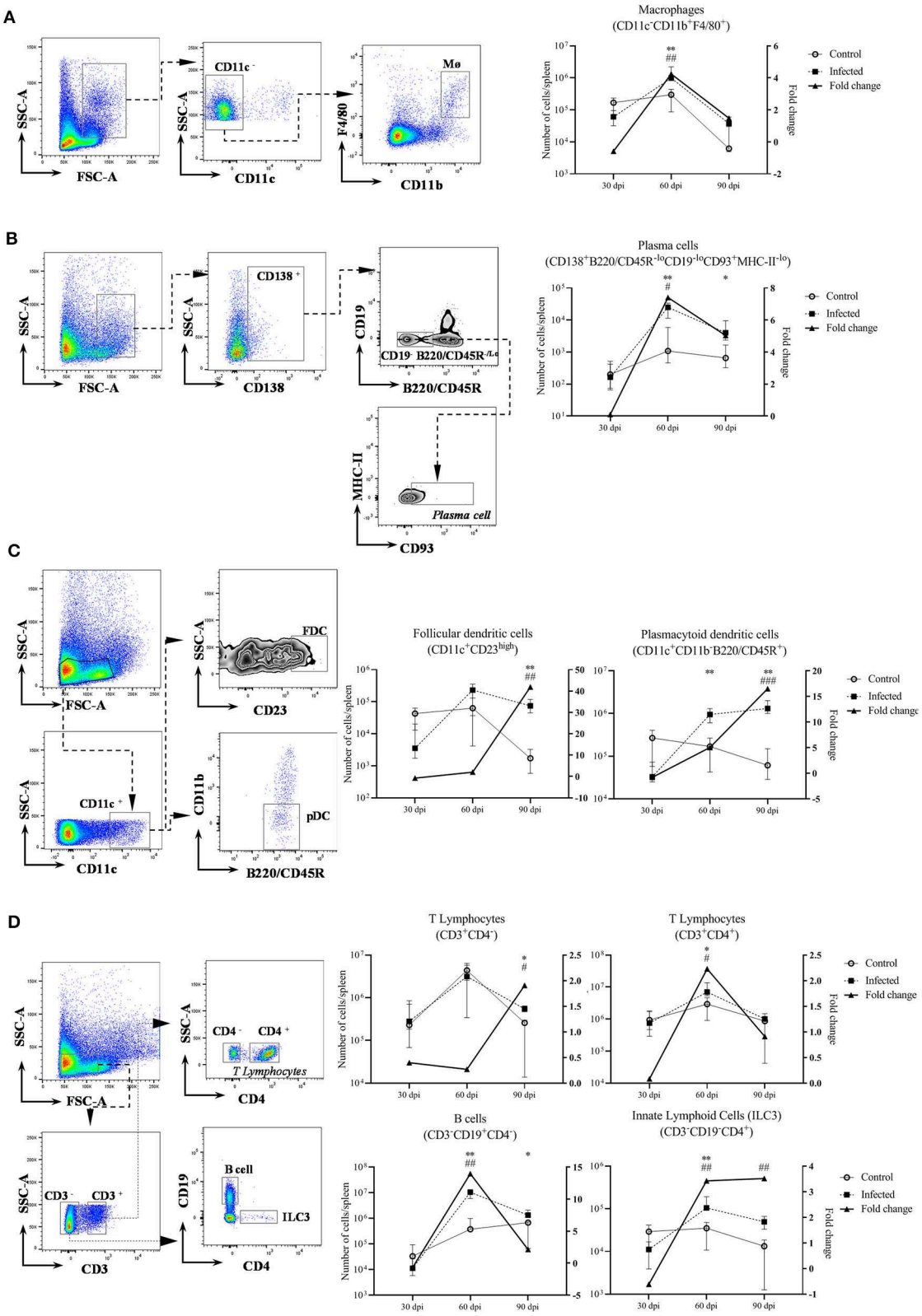

FIGURE 2 | Leukocyte populations in the spleen of uninfected and 108 *Leishmania*-infected mice. Leukocyte populations at 30, 60 and 90 dpi, five mice per group (left Y: absolute number of cells per spleen; right Y: fold change of infected/control. Graphs represent median and interquartile range). * = statistical difference

(Continued)

FIGURE 2 | between control and infected groups per time point, Mann-Whitney test. # = statistical difference between time points, ANOVA. **(A)** Macrophages ($CD11c^{-}CD11b^{+}F4/80^{+}$), $^{*}p = 0.007$; $^{\#}p = 0.005$, comparison between 30 dpi and 60 dpi; **(B)** plasma cells ($CD138^{+}B220/CD45R^{-}loCD19^{-}loCD93^{+}MHC{-}II^{-}lo$), 60 dpi $^{*}p = 0.007$, 90 dpi $^{*}p = 0.01$; $^{\#}p = 0.04$, comparison between 30 dpi and 60 dpi; **(C)** follicular dendritic cells ($CD11c^{+}CD23^{high}$), $^{*}p = 0.007$ and plasmacytoid dendritic cells ($CD11b^{-}CD11c^{+}B220/CD45R^{+}$), $^{*}p = 0.007$; $^{\#}p < 0.002$, comparison of 90 dpi with 30 dpi and 60 dpi; **(D)** T lymphocytes $CD3^{+}CD4^{+}$ and $CD3^{+}CD4^{-}$, $^{*}p = 0.03$; $^{\#}p = 0.01$, comparison between 30 dpi and 60 dpi; B cells ($CD3^{-}CD19^{+}CD4^{-}$) 60 dpi, $^{*}p = 0.007$ and 90 dpi, $^{*}p = 0.03$; $^{\#}p < 0.001$, comparison of 60 dpi with 30 dpi and 90 dpi; innate lymphoid cells ($CD3^{-}CD19^{-}CD4^{+}$), $^{*}p = 0.007$; $^{\#}p < 0.002$, comparison of 30 dpi with 60 dpi and 90 dpi.

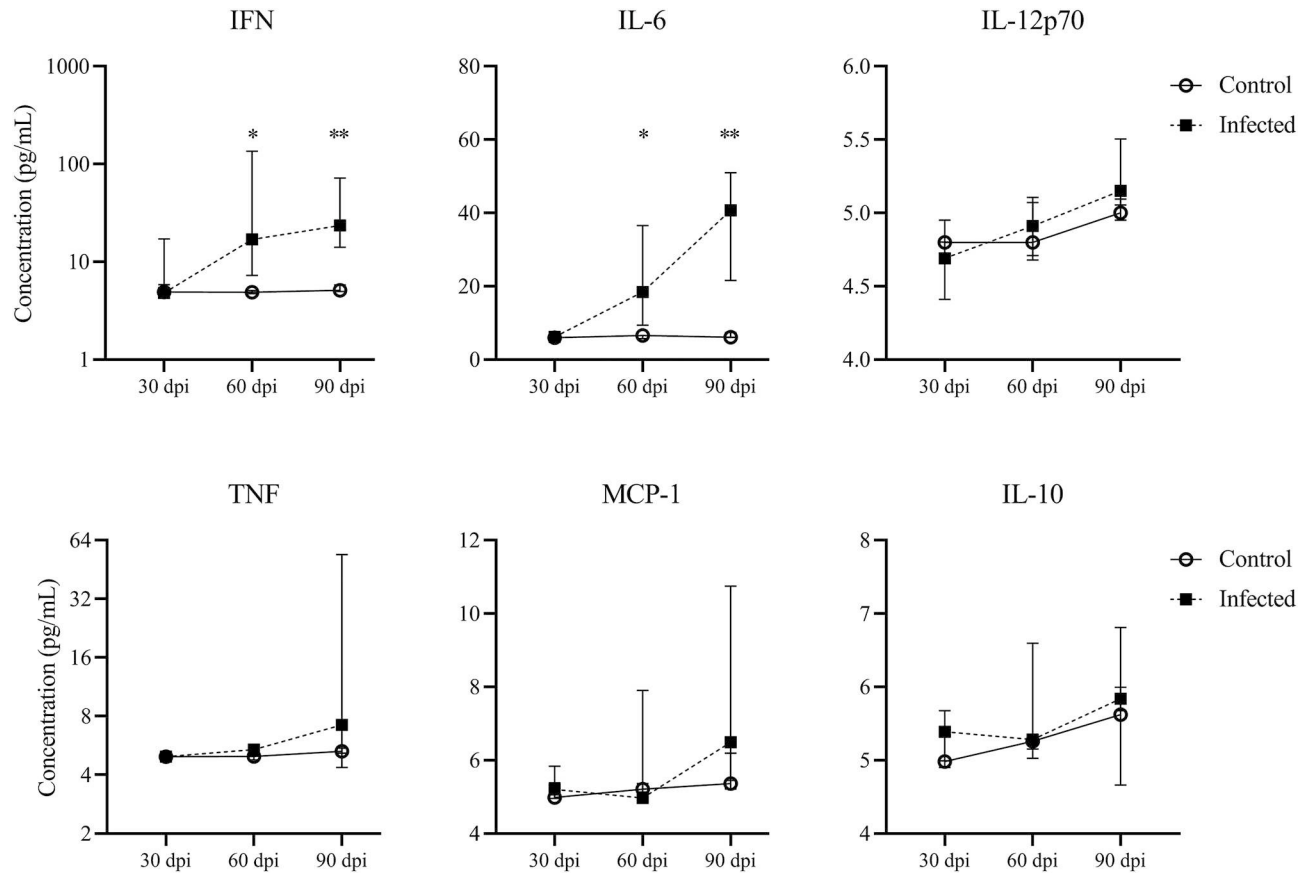

FIGURE 3 | Cytokine production by splenocytes. Concentrations of IFNγ, IL-6, IL-12p70, TNF, MCP-1 and IL-10 in *Leishmania*-stimulated spleen cells of mice at 30, 60 and 90 dpi, five mice per group. Graphs represent the median and interquartile range. Statistical differences of concentrations of IFN at 60 ($^{*}p$=0.03) and 90 dpi ($^{**}p = 0.007$) and IL-6 at 60 dpi ($^{*}p = 0.02$) and 90 dpi ($^{**}p = 0.007$), Mann-Whitney test.

Figure 1C). Hyperplasia of RP was observed in infected mice at 90 dpi (1 [0.7–1.2]) but not in the control mice (0 [0–0], $p = 0.002$]). Secondary lymphoid follicles with large germinal centers were more frequent in infected mice at 60 dpi (2 [1-2]) and 90 dpi (2 [1–3]) in comparison to the control groups (0 [−0.5–0.5], $p = 0.01$; 0 [−1–0], $p = 0.001$, 60 and 90 dpi, respectively). Lymphoid follicles were significantly increased at 90 dpi (2 [0.7–3], $p = 0.03$). Granulomas were only observed in infected mice at 60 dpi (1/7) and were more frequent and intense in infected mice at 90 dpi (**Figure 1B**) (6/6, $p = 0.001$). Only slight changes in the WP were observed in the animals infected with 10^7 promastigotes of *L. infantum* without statistical difference (data not shown).

Leukocyte Populations in the Spleen

Significant alterations in the leukocyte populations were detected at 60 dpi (**Figure 2**). Infected mice at 60 dpi presented with an increased number of macrophages, plasma cells, plasmacytoid dendritic cells (pDC), $CD4^{+}$ T lymphocytes and B cells. We also found an increased number of $CD3^{-}CD19^{-}CD4^{+}$ cells in infected mice at 60 dpi, with a suggestive phenotype of ILC3 (**Figure 2**). The fold change of FDC, pDC and $CD3^{+}CD4^{-}$ T lymphocytes progressively increased over time of infection. The number of B cells, pDC and plasma cells remained increased to the last time point (90 dpi) in comparison to control mice. Similar trends were observed by 60 dpi in an independent experiment following a 10^7 dose of *L. infantum*, including increased number

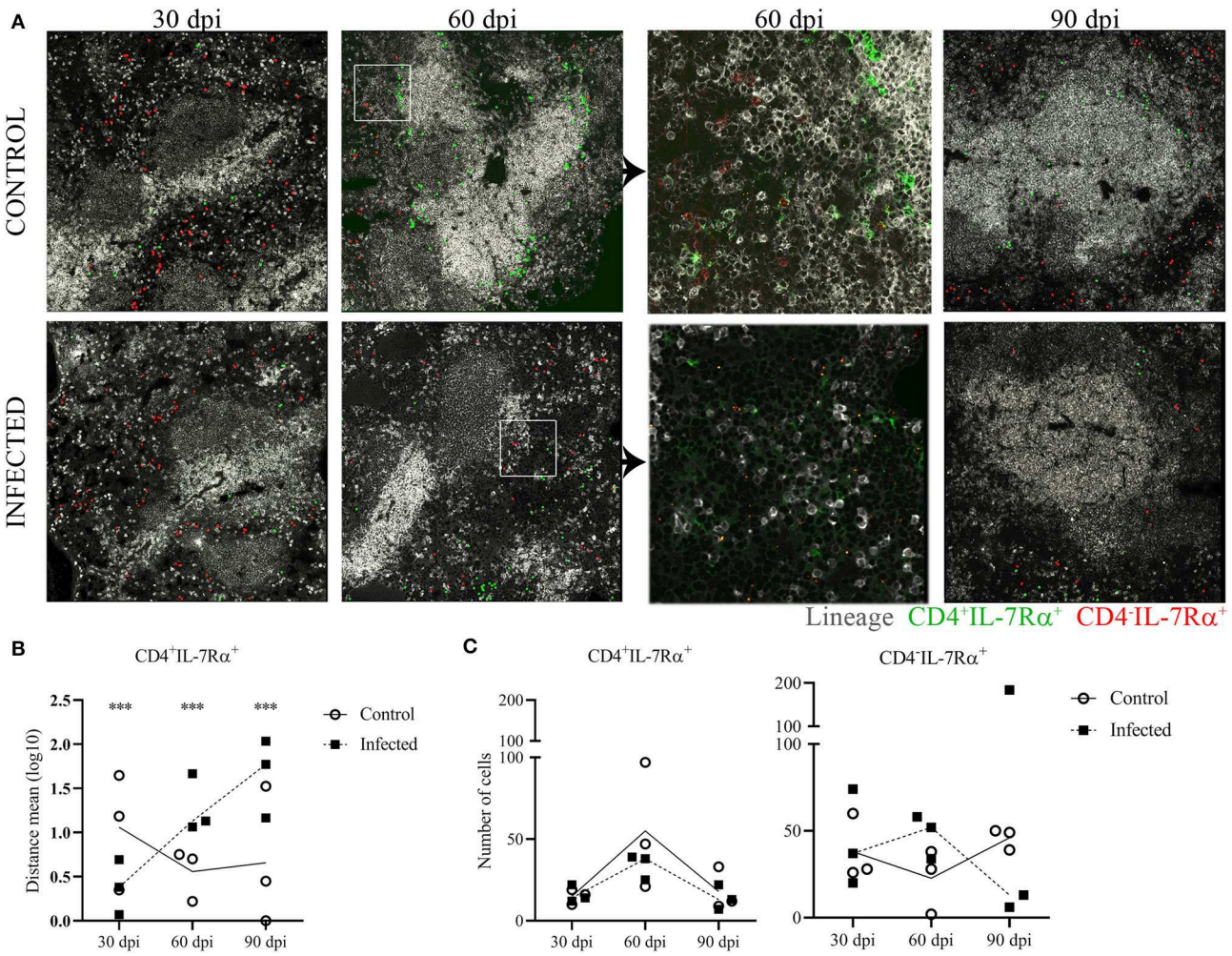

FIGURE 4 | Lymphoid tissue inducer cells distribution in splenic tissue. Representative spleen confocal photomicrographs of *L. infantum*-infected and uninfected (control) mice after 30, 60 and 90 dpi, three mice per group. **(A)** Mosaic of nine pictures at × 400 magnification, bars = 50μm. Lineage B220/CD11b/CD11c/CD3 gray staining, CD4 green staining and IL-7Rα$^+$ red staining. LineageCD4-IL-7Rα$^+$ (red dots) represents CD4$^-$ LTi cells and lineage$^-$CD4$^+$IL-7Rα$^+$ (green dots) represents CD4$^+$ LTi cells. **(B)** Log10 of distance means between the CD4$^+$ LTi cells and the edges of the white pulp in the spleen of three mice per group after 30, 60 and 90 dpi (***$p < 0.001$), Kruskal-Wallis test. **(C)** Number of LTi cell subsets assessed by histo-cytometry of spleens of three mice per group after 30, 60 and 90 dpi.

of suggestive CD3$^-$CD4$^+$ ILC3 at 60 dpi in infected mice (**Supplementary Figure 1**).

Cytokine Production by Splenocytes

Leishmania-stimulated spleen cells in the infected mice produced higher concentrations of pro-inflammatory cytokine IFN at 60 dpi (16.9 pg/mL [7.2–135.4]) and 90 dpi (23.5 pg/mL [14–72]) than control mice (4.8 [4.7–5], $p = 0.03$; 5.1 [4.9–5.8], $p = 0.007$, 60 and 90 dpi, respectively) (**Figure 3**). A progressive increase in IL-6 concentration was observed in infected mice from 60 dpi (18.4 [9.3–36.6]) to 90 dpi (40 [21.5–50.9]) in comparison to control mice (6.5 [5.6–6.5], $p = 0.02$; 7.3 [6.1–7.5], $p = 0.007$, 60 and 90 dpi, respectively) (**Figure 3**). We did not observe statistical differences in MCP-1, TNF, IL-12p70 or IL-10 concentrations between the control and infected groups at any time point (**Figure 3**).

Lymphoid Tissue Inducer Cells Distribution in Splenic Tissue

Due to the finding of an increased number of likely ILC3-LTi cells by flow cytometry at 60 days of infection, we further investigated the distribution of these cells in the spleen. The phenotype for the CD4$^-$ LTi subset was defined as lineage$^-$CD4$^-$IL-7Rα$^+$ (red dots) and for the CD4$^+$ LTi subset subtype as lineage$^-$CD4$^+$IL-7Rα$^+$ (green dots). The CD4$^-$ LTi cells were mainly located in the RP and similarly distributed in infected and uninfected animals (**Figure 4A**). In control mice, CD4$^+$ LTi cells appeared mainly distributed around the periarteriolar lymphoid sheath (PALS) (**Figure 4A**, gray staining). However, CD4$^+$ LTi cells were scattered in the RP in infected mice at 60 and 90 dpi as shown by the distance between the LTi cells and the edges of the WP (**Figure 4B**). Numeric differences of the LTi subsets were not evident in the

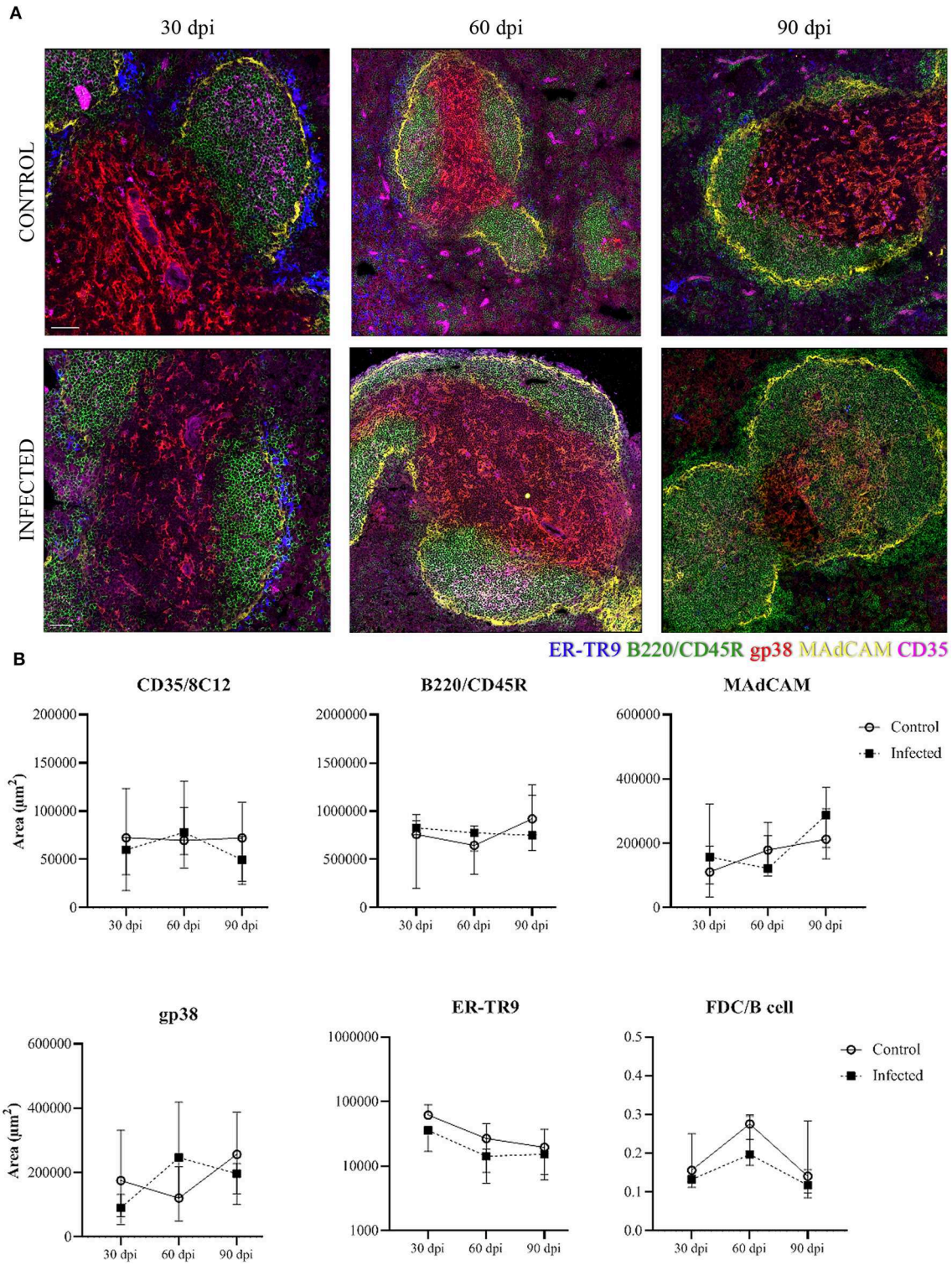

FIGURE 5 | Stromal cells in the splenic tissue. Representative spleen confocal photomicrographs of *L. infantum*-infected and uninfected (control) mice after 30, 60, and 90 dpi. **(A)** Mosaic of nine pictures at × 400 magnification, bars = 50 µm. Stromal cell populations are represented by marginal reticular ER-TR9^{+} cells (blue), marginal reticular MAdCAM^{+} cells (yellow), B220/CD45R^{+} B cells (green) and follicular dendritic CD35^{+}cells (magenta). **(B)** Quantification of positive area for stromal cell markers in the spleen after 30, 60, and 90 dpi. The graphs represent the median and interquartile range (Kruskal–Wallis test).

histological analysis between infected and uninfected animals (**Figure 4C**).

Stromal Cells Area Distribution in the Spleen

We analyzed the stromal cell distribution in the different compartments of the WP and the marginal zone. At 30 dpi a trend to a decrease of the marginal reticular ER-TR9$^+$ cells (blue) area was observed in infected mice in comparison to control mice (**Figure 5A**). The ER-TR9$^+$ cell area was similar between control and infected mice at 60 and 90 dpi (**Figure 5B**). For marginal reticular MAdCAM$^+$ cells (yellow) the area was not different between uninfected and infected mice. In the lymphoid follicles, B220/CD45R$^+$ B cell (green) area and FDC CD35$^+$ cell (magenta) area were not different between groups (**Figure 5**), nor was the ratio FDC/B cells in the lymphoid follicle (**Figure 5B**). The area of fibroblastic reticular gp38$^+$ cells in the PALS was not uniform across the different subjects (**Figure 5**).

DISCUSSION

In this work we studied the kinetics of spleen remodeling in late stages of chronic VL using an experimental model of infection in BALB/c mice. Early changes in cell populations and cytokines have been previously reported (16, 22, 23, 38). However, little is known about the progressive morphological changes that result in extensive disruption of the WP in the late stages of the disease. We observed that BALB/c mice infected with 10^8 promastigotes of *L. infantum* developed a progressive follicle hyperplasia that became evident at 60 dpi. This spleen hyperplasia was associated with an increase in macrophages, B cells, plasma cells, follicular and plasmacytoid dendritic cells as well as ILC3-LTi cells. The distribution of LTi cells was also altered on the 60th day of infection. The WP area was significantly reduced at 90 dpi and Interferon gamma and IL-6 concentrations were consistently elevated in the spleen of the infected animals.

Spleen Remodeling in the BALB/c Model of VL

Hyperplasia followed by atrophy and WP disruption is common in severe terminal leishmaniasis in some susceptible species such as humans and dogs (14, 39–41). Although hyperplasia and important cell changes took place in the mice in this study, only a decrease in WP area and a slight morphological disorganization of lymphoid tissue was observed, even though a large number of parasites were injected. Alterations in the splenic microenvironment have been reported upon natural *L. infantum*/*L. donovani* infection in human and dogs and in BALB/c mice and hamsters that were experimentally infected (11, 16, 22, 23, 38).

Substantial changes in cell populations in marginal zone and WP take place as early as 14 to 28 days of infection (21, 23, 33, 42). In this study we also observed changes in the ER-TR9$^+$ stromal cell network in the marginal zone as well as WP hyperplasia associated with an increased number of macrophages, B cells, plasma cells, follicular and plasmacytoid dendritic cells and ILC3-LTi-like cells identified by flow cytometry from 30 to 90 dpi. A possible explanation for the late presentation of the splenic changes in our study is the intraperitoneal route used for infection. In most of the other studies the intravenous route was used (21–23). In this work, it seemed that some of the early alterations of the marginal zone and the WP were restored in later stages of the disease. It is not known how these initial and reversible changes may contribute to the late and potentially irreversible WP disruption. However, a decrease of lymphoid follicle size and lymphoid atrophy, following a period of lymphoid hyperplasia, seems to be dependent on the inoculum size and to remain through the subsequent course of the disease (11, 23). In fact, in this study, infection with 10^7 promastigotes by intraperitoneal route only led to mild alterations in cell populations in the spleen (**Supplementary Figure 1**) and did not produce an increase in spleen size or weight. Although variations in cell counts occur in uninfected mice, the results of the two independent experiments with different inoculum sizes are similar. With a high parasite inoculum, important splenic changes were observed. Additionally, CD4$^+$ LTi cells changed their distribution between 30 and 60 days of infection, as observed by histo-cytometry. The changes in the number and distribution of the cell populations, the peak of parasite burden and splenomegaly were all consistently present at 60 dpi, a key time-point in the progression of the disease. In fact, a complete reorganization of lymphoid tissue and vascular network may be involved in splenomegaly (22). In spite of all these changes a complete disruption of WP was not observed in this model of experimental murine VL.

These observations are consistent with the reported course of *L. infantum* infection in BALB/c mice, where splenomegaly and a paucity of clinical signs of VL are observed (22, 43). The parasite burden in the spleen reached a plateau at day 90 of infection, associated with a Th1 cytokine expression pattern. Together with the absence of a number of other clinical signs of disease apart from spleen enlargement, and only slight disorganization of the splenic structure, protective immunological signaling pathways may take place in murine *L. infantum* infection, as reviewed by Rodrigues et al. (44). The dynamics of ER-TR9$^+$ network disruption and possible subsequent restoration, together with the LTi cell redistribution, suggests that control of spleen remodeling is also present in late stages of murine VL.

Lymphoid Tissue Inducer Cells and Spleen Remodeling in BALB/c Mice VL

Evidence suggests that spleen lymphoid tissue organizers cells as well as LTi cells continue to play a role in WP maintenance in neonatal and adult life (30, 31). These cells persist around PALS and interfollicular areas (45) and may participate in WP

regeneration after damage (46). In this study we show that the proportion of the $CD4^+$ LTi subset was increased in the spleen between 60 to 90 dpi. These cells also were differentially distributed in the spleens of infected compared to uninfected mice. In most of the animals of the control group, $CD4^+$ LTi cells were spotted surrounding the PALS, such as described for normal resting spleen (45). In the infected animals, $CD4^+$ LTi cells showed no longer the same distribution, and were instead scattered in the RP at 60 and 90 dpi. Although the quantitative changes of $CD4^+$ LTi cells observed by flow cytometry have been confirmed in two different experiments with different parasite inoculum, it was not possible to confirm these quantitative changes in upon histo-cytometry analysis. A possible explanation is that histo-cytometry (by using only 4 μm thick transversal sections of the spleen) may not be as sensitive as flow cytometry analysis (using 10^5 dispersed cells) for detecting small variations in the number of cells represented in small quantities. However, histo-cytometry analysis allowed visualization of LTi subsets distribution in spleen compartments. Nevertheless, we cannot exclude the possibility that LTi cells play a role in the WP regeneration in severe VL. In fact, it has been shown that LTi cells still play a role in spleen embryogenesis although this role may not be critical (30). An increase in the proportion of LTi cells was also observed by Scandella et al. (32) in the spleen of mice infected with lymphocytic choriomeningitis virus. The increase in the proportion of these cells preceded the restoration of spleen histological structure. Vivier et al. (47) suggested that LTi cells also have a preventive role in lymphoid tissue damage. However, Dalton et al. (33) demonstrated using B6.Rorc$^{-/-}$ → B6.CD45.1 chimeric LTi cell-deficient mice that WP regeneration in mice infected with *L. donovani* and treated with Sunitinib maleate could be independent of LTi cells. Although participation of LTi cells was not crucial to the WP regeneration in the work by Dalton et al. (33), their participation is not excluded in WP preservation in VL.

The data shown in this work together with the reported data in the literature on murine VL suggests that mice infected with *L. infantum* or *L. donovani* present some degree of spleen disorganization between 14 and 30 dpi evidenced by loss of marginal zone macrophages, disruption to both the FDC network in B cell follicles and transient disruption of the ER-TR9$^+$ stromal cell network (16, 21). These alterations are followed by changes in the number and distribution of LTi cells and structural preservation of the WP up to 90 dpi.

Further studies are necessary to confirm the protective role played by LTi cells in preventing changes in the spleen upon VL and the mechanisms involved in this process.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Research Ethics Committee of Instituto Gonçalo Moniz (IGM-FIOCRUZ, license nos. 004/2013 and 017/2015).

AUTHOR CONTRIBUTIONS

CM and WS came up with the ideas for this experiment. CM, MH, BM, and JF planned and carried out the flow cytometry experiments. JK and RM planned the LTi and stromal cells panel. CM conducted the experiments and analysis. MS performed the qPCR for parasite burden. BB performed the morphometric analysis and obtained the serological evidence of infection. GM processed the tissues and prepared the slides. LF, WS, and CM performed the histopathological analysis. RM and WS supervised the project. CM and WS wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00653/full#supplementary-material

Supplementary Figure 1 | Leukocyte populations in the spleen of uninfected and 10^7 *Leishmania*-infected mice. Leukocyte populations at 30, 60, 90, 120, and 150 dpi (left Y: absolute number of cells; right Y: fold change of infected/control. Graphs represent median and interquartile range). * = statistical difference between control and infected groups per time point, ANOVA. # = statistical difference between time points, ANOVA. Follicular dendritic cells ($CD11c^+CD23^{high}$), #$p = 0.03$, comparison between 90 dpi and 120 dpi; plasmacytoid dendritic cells ($CD11b^-CD11c^+B220/CD45R^+$), #$p = 0.03$, comparison of 90 dpi with 30 dpi; innate lymphoid cells ($CD3^-CD4^+$), *$p = 0.03$; #$p < 0.001$, comparison of 60 dpi with 30, 90, 120, and 150 dpi.

Supplementary Table 1 | Anti-mouse antibodies for immunophenotyping.

Supplementary Table 2 | Evidence of infection and clinical evaluation of the mice infected with 10^7 promastigotes of *L. infantum*.

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REVIEW
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Buruli ulcer: The Efficacy of Innate Immune Defense May Be a Key Determinant for the Outcome of Infection With *Mycobacterium ulcerans*

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Buruli ulcer (BU) is a neglected, tropical infectious disease of the skin and the subcutaneous tissue caused by *Mycobacterium ulcerans.* This pathogen has emerged as a new species from a common ancestor with *Mycobacterium marinum* by acquisition of the virulence plasmid pMUM. The plasmid encodes enzymes required for the synthesis of the macrolide toxin mycolactone, which has cytotoxic and immunosuppressive activities. In advanced BU lesions, extracellular clusters of *M. ulcerans* reside in necrotic subcutaneous tissue and are protected from infiltrating leukocytes by the cytotoxic activity of secreted mycolactone. Several lines of evidence indicate that elements of the innate immune system eliminate in many cases the initial inoculum before bacterial clusters can form and that therefore exposure to *M. ulcerans* leads only in a minority of individuals to the characteristic chronic necrotizing BU lesions. It is assumed that phagocytes play a key role in early host defense against *M. ulcerans*. Antibodies against bacterial surface structures seem to have less potential to enhance innate immunity than T_H1 cell responses. Precise innate and adaptive immune effector mechanisms leading to protective immunity are however unclear, complicating the development of effective vaccines, the most desired solution to control BU. The tuberculosis vaccine *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) has limited short-term protective activity against BU. Whether this effect is due to the broad antigenic cross-reactivity between *M. bovis* and *M. ulcerans* or is at least partly mediated by a non-specific enhanced responsiveness of innate immune cells to secondary stimulation, recently described as "trained immunity" or "innate immune memory" is unknown but has major implications for vaccine design. Current vaccine research and development activities are focusing on recombinant BCG, subunit vaccines with selected *M. ulcerans* proteins, and the neutralization of mycolactone.

Keywords: *Mycobacterium ulcerans*, skin neglected tropical disease, mycolactone, *Mycobacterium marinum*, zebrafish

INTRODUCTION

Innate immunity constitutes the first line of host defense against potentially pathogenic microbial invaders. It comprises physical and various chemical barriers, including antimicrobial proteins, to prevent entry into the host, as well as innate humoral (such as the alternative complement pathway) and cellular defense mechanisms that come into play if the epithelial barriers are breached. Innate immune cells, such as macrophages, neutrophils, and dendritic cells express pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) that can detect so called pathogen-associated molecular patterns (PAMPs) common to many microorganisms, or damage-associated molecular patterns (DAMPs) of host molecules released by infected or dying cells. Upon recognition, these "sensor cells" can either act directly as effectors, phagocytosing and degrading the pathogens or indirectly, by producing inflammatory mediators, such as cytokines and chemokines that can attract and activate other immune cells. If the infection persists, phagocytes also connect the innate with the adaptive immune system by presenting antigens to antigen-specific T and B cells. Evidence has been accumulating in recent years that after infection or vaccination, innate immune cells display changes in their transcription programs and cell physiology, which may lead to transiently increased responsiveness upon secondary stimulation by microbial pathogens, a phenomenon termed "trained immunity" (Netea et al., 2011, 2016).

Pathogenic mycobacteria, including *Mycobacterium tuberculosis* and its near relative *M. marinum* (Stinear et al., 2008) have developed mechanisms to subvert the innate immune response. They can establish residence inside host macrophages and use host granulomas – organized immune cell aggregates, characterized by the presence of mature macrophages, that can contain but fail to eradicate infection foci – for their expansion and dissemination during the innate phases of infection (Ramakrishnan, 2013). *M. marinum*, which causes a granulomatous, tuberculosis-like disease in ectotherms (Tobin and Ramakrishnan, 2008), has gained popularity as a model organism for mycobacterial infections and has thus been extensively studied. Amongst different model systems, experimentally infected zebrafish (*Danio rerio*) embryos and early swimming larvae have become a powerful resource to study contributions of innate immune responses to combat mycobacterial infections (Davis et al., 2002). Due to the absence of an adaptive immune system at these early developmental stages, the zebrafish model has significantly advanced our understanding of innate host defense against mycobacterial infections.

In comparison, little is known on early interactions of the immune system with *M. ulcerans*, which causes the chronic, necrotizing skin disease Buruli ulcer (BU) and has emerged as a new species from a common ancestor with *M. marinum* by acquisition of a virulence plasmid (pMUM) and subsequent reductive evolution (Doig et al., 2012). Despite more than 98% overall nucleotide identity between the genomes of the two pathogens (Stinear et al., 2007), *M. ulcerans* has developed a markedly different strategy for immune evasion, primarily due to the pMUM-mediated ability to produce mycolactone, a diffusible cytotoxic and immunosuppressive macrolide toxin (George et al., 1999). While an early intra-macrophage growth phase of *M. ulcerans* has been postulated (Coutanceau et al., 2005; Silva et al., 2009), in advanced disease, *M. ulcerans* bacilli are predominantly found extracellularly in the necrotic core of BU lesions, that is devoid of living, infiltrating immune cells (Ruf et al., 2017). Infection with *M. ulcerans* can either be contained by the immune system as indicated by reports of spontaneous healing (Marion et al., 2016a; O'Brien et al., 2019) and of *M. ulcerans*-specific immune responses in exposed, but healthy individuals (Gooding et al., 2001; Diaz et al., 2006; Yeboah-Manu et al., 2012), or can lead to serious dermatologic manifestations and chronic necrotizing disease (Pluschke and Röltgen, 2019). Understanding of early immune mechanisms involved in the diverse outcome of infection with *M. ulcerans* is however incomplete. In this review article, we compare the pathogenesis of *M. ulcerans* and *M. marinum* infections and summarize current data on innate immune mechanisms against infection with *M. ulcerans*. Knowledge on correlates of protection against BU has important implications for the rational design of a vaccine – the ideal solution to control the disease as discussed at the end of this article.

Mycobacterium ulcerans HAS EVOLVED FROM AN *M. marinum*-LIKE PROGENITOR

Common Ancestry. . .

Buruli ulcer mainly affects inhabitants of rural, focal areas in West and Central Africa and yet the host range of *M. ulcerans* is broad (Röltgen and Pluschke, 2015). Apart from human BU lesions that most commonly involve the extremities, *M. ulcerans* has been isolated from lesions of other mammals in Australia (Fyfe et al., 2010) and from diseased fish and frogs around the world (Trott et al., 2004; Rhodes et al., 2005; Ranger et al., 2006; Stragier et al., 2008). Based on comparative genomic data, two major lineages of mycolactone-producing mycobacteria (MPM) have been distinguished, the classical lineage isolated from humans in Africa, Australia, and Papua New Guinea, and from other mammals and the ancestral lineage, which can be subdivided into at least two deep rooted sub-lineages; human disease isolates from Japanese patients (also designated *Mycobacterium ulcerans* subsp. *shinshuense*) and strains isolated from humans in the Americas and from ectotherms (Käser et al., 2007; Doig et al., 2012). Genomic data indicate that MPM have emerged only once through the acquisition of pMUM and therefore all MPM should be designated *M. ulcerans* (Yip et al., 2007; Pidot et al., 2010).

Proximity to aquatic habitats, and particularly activities within stagnant or slow flowing water bodies have been identified as a common risk factor for human BU in different geographical areas (Raghunathan et al., 2005; Kenu et al., 2014; N'krumah et al., 2017; Maman et al., 2018). Knowledge on transmission pathways and reservoirs of *M. ulcerans* is still fragmentary, but inoculation of the bacteria into the skin by postulated insect vectors or

from environmental reservoirs via skin trauma is hypothesized. Association of *M. ulcerans* with aquatic environments has long been suspected due to its emergence from *M. marinum*, a ubiquitous pathogen of fish and other ectotherms. Occasional human *M. marinum* infections, which most commonly involve fingers and/or hands, are nowadays mainly connected with exposure to fish tanks, handling of fish, and boating/fishing-related activities (Johnson and Stout, 2015). Transmission of *M. marinum* is thought to occur through inoculation of the bacteria into the skin via cuts or lacerations (Petrini, 2006).

Mycobacterium ulcerans and *M. marinum* grow optimally at around 30°C and poorly at 37°C and above, which may at least in part explain their skin tropism and limited systemic dissemination. Both pathogens belong to the group of slow-growing mycobacteria, whereby the generation time in microbial culture medium of *M. ulcerans* (several days) is considerably longer than that of *M. marinum* (∼4–6 h) (Clark and Shepard, 1963; Marsollier et al., 2004). A mean incubation period of 4.5 months has been determined for *M. ulcerans* infections in a study on BU patients in Australia (Loftus et al., 2018). The incubation period of *M. marinum* is estimated to be ∼3 weeks, but can be up to several months long (Jernigan and Farr, 2000). Infections with both pathogens may resolve spontaneously by activities of the immune system but require long-term antibiotic treatment when established. Current WHO treatment recommendations for *M. ulcerans* infections comprise a combination therapy with rifampicin and clarithromycin (or streptomycin) for 8 weeks and surgery if indicated (World Health Organization, Global Buruli Ulcer Initiative, 2004; Phillips et al., 2020). There are no clinical trials to guide optimal management of *M. marinum* infections, but treatment with two active agents (clarithromycin/azithromycin, ethambutol, or rifampicin) for 3–4 months with adjunct surgical debridement for invasive infections has been reported (Griffith et al., 2007). Person to person transmission of *M. ulcerans* and *M. marinum* infection is considered unlikely. Thus, at first glance, characteristics of *M. ulcerans* and *M. marinum* and of the infections they cause seem very similar. Marked differences emerge however when comparing pathogenesis and host responses evoked by the two related mycobacterial species.

. . . but Vastly Different Pathogenesis

Infection with *M. ulcerans* initially produces subcutaneous nodules or papules with a necrotic core or less frequently plaques and edema with laterally extended destruction of subcutaneous tissue. As the early stages of the disease are often painless, patients tend to report late to health facilities, when severe skin and soft tissue destruction has started. In advanced stages of the infection, the epidermis overlying the necrotic deeper layers of the skin sloughs off, and chronic ulcers with undermined edges develop. Histopathologic hallmarks of BU lesions are a progressive contiguous coagulative necrosis of the deep dermis and subcutaneous fat tissue with clusters of acid-fast bacilli (AFB), but no viable infiltrating leukocytes in the core of necrotic areas. In patients with chronic non-healing BU, squamous cell carcinoma may develop (Evans et al., 1999). Human *M. marinum* disease, often referred to as "fish tank granuloma" is commonly limited to a single, nodular cutaneous lesion, but can progress to invasive disease such as tenosynovitis and less frequently arthritis and osteomyelitis (Aubry et al., 2002; Johnson and Stout, 2015). The histopathological spectrum of *M. marinum* infections is broad and depends on the course and stage of the disease (Travis et al., 1985; Sia et al., 2016). Features in skin biopsies range from poorly formed granulomas with loose infiltrates of epithelioid macrophages, scattered multinucleated giant cells, and lymphohistocytic dermal inflammation to well-formed granulomas with circumscribed, nodular macrophage infiltrates. Granulomas frequently contain a central necrotic core, often surrounded by mixed inflammation and granulation tissue (Sia et al., 2016). In deep soft tissue and synovial biopsies, moderately well-formed, non-caseating or necrotizing, suppurative granulomas with giant cells are seen (Beckman et al., 1985; Sia et al., 2016). Acute and chronic synovial inflammation characterized by a paucity of plasma cells, often accompanied by synovial hyperplasia, and fibrin exudation into the synovial space has been reported (Beckman et al., 1985; Travis et al., 1985; Sia et al., 2016). The normal synovial architecture may be replaced by extensive granulation tissue (Travis et al., 1985). In both skin and synovial samples, bacilli are not easily detected as they reside highly localized in necrotic, suppurative cores of the granuloma (Sia et al., 2016). Variation in pathogenicity may also be related to genetic differences, as *M. marinum* strains exhibit extensive genomic diversity (van der Sar et al., 2004; Broutin et al., 2012). Despite an overwhelming sequence similarity of 98% between *M. ulcerans* and *M. marinum*, explanations for differences in pathogenesis and immune defense can be found in the gene content of their genomes.

It Is Written in the Genes. . .

Mycobacterium ulcerans has acquired both a virulence plasmid, encoding genes for the biosynthesis of the unique macrolide toxin mycolactone, and insertion sequence (IS) elements that have mediated extensive loss of DNA. Whereas the genome of *M. marinum* strain M encompasses a 6.6 Mb circular chromosome with 5424 coding sequences (CDS), and 65 pseudogenes (Stinear et al., 2008), the genome of the *M. ulcerans* classical lineage reference strain Agy99 is considerably smaller, comprising the virulence plasmid pMUM of 174 kb, and a 5.6 Mb circular chromosome with 4160 CDS, and 771 pseudogenes (Stinear et al., 2007). Production of the macrolide toxin mycolactone was likely the key factor enabling the evolution of *M. ulcerans*.

Mycolactone consists of a conserved 12-membered lactone ring with a C-linked upper side chain and a less conserved lower C5-O-linked polyunsaturated acyl side chain (George et al., 1999). The lower side chains of the mycolactone variants produced by different *M. ulcerans* sub-lineages are structurally diverse (**Figure 1**) and differ in toxic potency (Scherr et al., 2013). In addition to potent cytotoxic activity, mycolactone exhibits analgesic and immunosuppressive properties at sub-toxic concentrations (Phillips et al., 2009; Hall et al., 2014; Marion et al., 2014; Guenin-Macé et al., 2019). Low nanomolar concentrations of mycolactone cause *in vitro* apoptosis within 2–5 days in a wide range of mammalian cells (Bozzo et al., 2010;

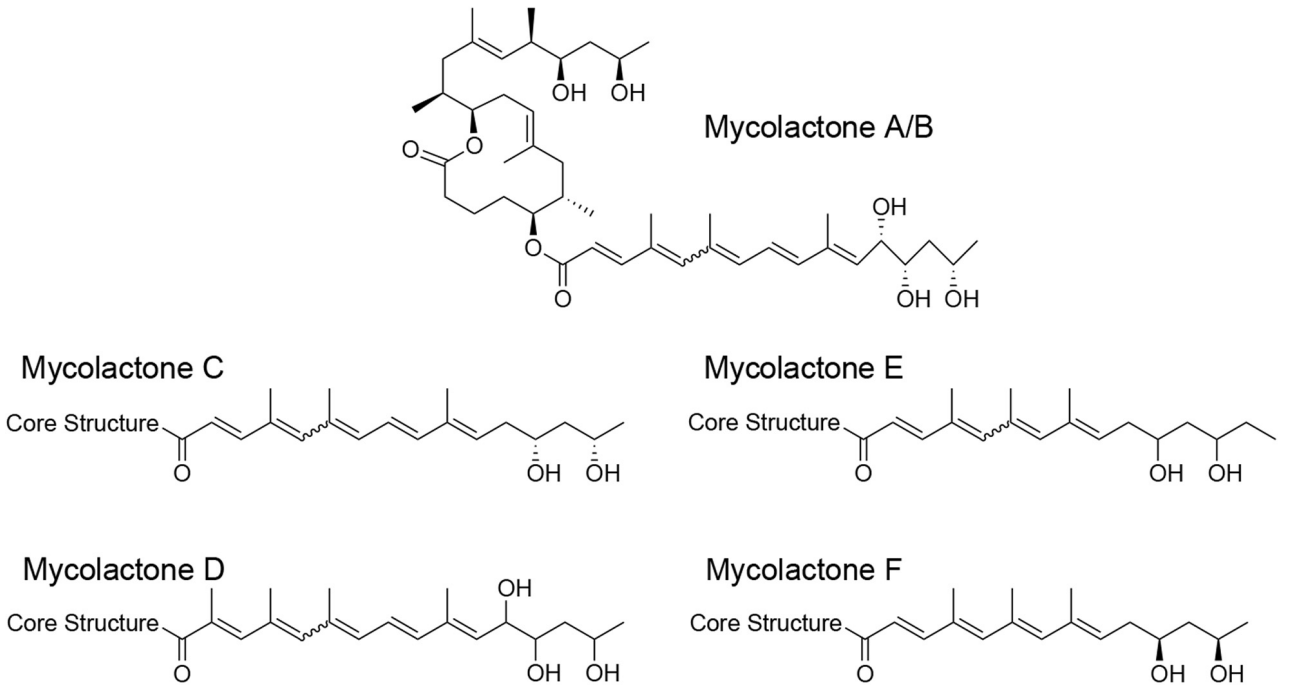

FIGURE 1 | Structure of mycolactone variants. Mycolactone congeners were shown to be produced by *M. ulcerans* strains isolated from BU patients in Africa (mycolactone A/B), Australia (mycolactone C), and China (mycolactone D) and from fish and frogs of different geographical origin (mycolactone E and F).

Scherr et al., 2013; Guenin-Mace et al., 2015; Ogbechi et al., 2015). The secreted toxin seems to diffuse passively through mammalian cell membranes and to promote Bim-dependent apoptosis through the Akt-FoxO3 axis, as demonstrated by the absence of necrotic BU lesions in *M. ulcerans*-infected Bim knock-out mice (Bieri et al., 2017). In contrast, necrotizing lesions with features of human BU are caused by injection of mycolactone or mycolactone-producing *M. ulcerans* into the skin of wild-type mice and other experimental animals (George et al., 2000; Oliveira et al., 2005; Sarfo et al., 2013). Among several distinct proposed molecular mechanisms for the mode of action of mycolactone, selective inhibition of the Sec61 translocon-mediated co-translational transport of secretory proteins into the ER seems to play the key role (Hall et al., 2014; Baron et al., 2016). The inhibition of protein translocation leads to pronounced proteomic changes and an integrated cellular stress response that ultimately seems to drive Bim-dependent apoptosis. In addition, downregulation of cytokines and chemokines at sub-toxic concentrations has strong immunosuppressive effects. The Wiskott–Aldrich syndrome protein (WASP) family has been proposed as another molecular target of mycolactone (Guenin-Macé et al., 2013). Binding of the toxin to WASP/N-WASP appears to lead to uncontrolled assembly of actin and defects in cell adhesion, which may obstruct innate cellular immune responses.

One additional important difference in the gene content of the *M. marinum* and *M. ulcerans* genomes is the repertoire of ESX secretion systems and of PE/PPE proteins. The mycobacterial ESX loci are large gene clusters that encode a type VII secretory apparatus required for export of members of the 6-kDa early secreted antigenic target (ESAT-6) protein family together with other effector proteins across the complex cell envelope. Genes encoding ESAT-6 and the 10 kDa culture filtrate protein (CFP-10) are located directly adjacent to each other and are co-transcribed. The genome of the *M. tuberculosis* H37Rv strain contains 11 pairs of these *esx* tandem genes (Cole et al., 1998). *M. marinum* has 29 *esx* genes within five ESX loci (Stinear et al., 2008). The prototypical ESX-1 system is a major virulence determinant in *M. tuberculosis* and *M. marinum*, triggering granuloma formation as well as intercellular bacterial spread between macrophages (Volkman et al., 2004). The absence of a 9.5 kb genomic region across all *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) strains, termed Region of Difference 1 (RD1), located in the ESX-1 locus, is a major molecular determinant underlying BCG attenuation. *M. marinum* carries a partial duplication of the *esx-1* gene cluster, resulting in more than one copy of several genes including *esxA* (ESAT-6) and *esxB* (CFP-10). On the other hand, *M. ulcerans* has retained only 13 *esx* genes and three intact ESX loci. Disruption of the ESX-1 locus in *M. ulcerans* classical lineage strains, which led to the abolishment of ESAT-6 and CFP-10 secretion (Huber et al., 2008), may contribute to the predominantly extracellular location of *M. ulcerans* bacilli (Stinear et al., 2007).

PE/PPE proteins, found mostly in slow-growing pathogenic mycobacteria, are characterized by conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs at the N-termini. It has been reported that certain ESX secretion systems mediate the

secretion of several PE/PPE proteins (Abdallah et al., 2009; Shah et al., 2015). The genome of the *M. tuberculosis* H37Rv strain contains 99 *pe* and 69 *ppe* genes, but this number can vary for different *M. tuberculosis* isolates (Fishbein et al., 2015). The *M. marinum* genome codes for 175 PE and 106 PPE proteins, whereas *M. ulcerans* has preserved only 70 intact *pe* and 46 *ppe* genes. The function of PE and PPE proteins is still enigmatic, but the limited data available suggest that they are important for mycobacterial virulence. Some of the PE proteins from *M. marinum* are thought to be involved in modulating the macrophage environment (Ramakrishnan et al., 2000; Tiwari et al., 2012; Fishbein et al., 2015). Compared to *M. marinum, M. ulcerans* has thus both gained a major virulence determinant – mycolactone and lost several other virulence factors mainly associated with the intracellular lifestyle of its ancestor. The following two paragraphs illustrate how these genomic differences are reflected in diverse interactions of *M. marinum* and *M. ulcerans* with the innate immune system.

HOST-MYCOBACTERIUM INTERACTIONS – INSIGHTS INTO EARLY INFECTION EVENTS FROM A ZEBRAFISH MODEL

In the past decades, *M. marinum* has become a model organism to study fundamental mechanisms of mycobacterial pathogen-host interactions. Zebrafish are naturally susceptible to *M. marinum* and upon infection develop organized granulomas similar to those caused by *M. tuberculosis* (Westerfield, 2000; Swaim et al., 2006). Zebrafish embryos and early swimming larvae are a powerful means to dissect innate immune responses to *M. marinum,* as at these early developmental stages, they rely solely on innate immune mechanisms mediated by macrophages and neutrophils, and lack the elements of adaptive immunity (Davis et al., 2002). In this paragraph, we describe how the genetic tractability and optical transparency of zebrafish embryos have enabled a variety of elegant experimental approaches to study early *M. marinum* infection events *in vivo*.

First Hours After Infection

Real-time imaging of zebrafish embryos revealed that after intravenous injection of *M. marinum*, blood macrophages immediately take up mycobacteria and extravasate into diverse tissues. Strikingly, recruitment of macrophages within 6 h after injection of *M. marinum* into the zebrafish hindbrain ventricle – an isolated cavity devoid of macrophages in the absence of pathogens – demonstrated redirection of normal migration and differentiation of embryonic macrophages (Davis et al., 2002). Both live and heat-killed *M. marinum* seem to be able to recruit phagocytes (heat-killed mycobacteria are subsequently readily degraded after phagocytosis), suggesting that cell wall lipids or heat-stable proteins stimulate migration (Clay et al., 2007). It has been postulated that *M. marinum* is able to evade microbicidal effects of TLR-activated macrophages by masking PAMPs with cell-surface associated lipids, and instead recruits permissive macrophages via an alternative pathway. In infected macrophages, the production of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β is upregulated (Cambier et al., 2014). Studies involving the depletion of macrophages in zebrafish embryos illustrated that macrophages are required to restrict proliferation of *M. marinum* and thus constitute only a suboptimal growth niche for the bacilli. They do however play an important role in the dissemination of mycobacteria into the tissues (Clay et al., 2007).

Migration Into Tissue and Granuloma Formation

After phagocytosing the *M. marinum* bacilli, macrophages migrate into deeper tissue, where they begin to form dynamic, granuloma-like aggregates, becoming visible three days after intravenous inoculation. Aggregated cells display euchromatic nuclei characteristic of activated macrophages and either tightly apposed cell membranes or indistinct cell boundaries, distinctive features of epithelioid cells and multinucleated giant cells in mature granulomas. *M. marinum* is found both intracellular, sequestered by the cellular aggregates, or extracellular, in necrotic (caseous) foci (Davis et al., 2002). TNF was found to be a key effector molecule required for the maintenance of granuloma integrity. Ablation of TNF signaling in mutant zebrafish embryos causes both accelerated intracellular bacterial growth and granuloma formation, followed by increased macrophage death and necrotic breakdown of granulomas with resultant exuberant growth of extracellular mycobacteria (Clay et al., 2008). Interestingly, granuloma formation is generally associated with accelerated bacterial proliferation, plateauing only after several weeks with the onset of adaptive immunity, indicating that nascent granulomas promote mycobacterial expansion (Volkman et al., 2004). Indeed, zebrafish studies have illustrated that granuloma-forming processes are mediated through mycobacterial virulence factors. By a mechanism requiring the mycobacterial RD1/ESX-1 secretion system, new macrophages are recruited to the granuloma via chemotactic signals, and phagocytose infected macrophages undergoing apoptosis, leading to rapid, iterative expansion of the number of infected macrophages and thereby to an increase in the bacterial burden (Davis and Ramakrishnan, 2009). The secretory protein ESAT-6 seems to play a central role in these chemotactic effects by inducing matrix metalloproteinase-9 (MMP-9) expression in epithelial cells surrounding the granuloma. In turn, MMP-9 is thought to enhance recruitment of macrophages to the growing granuloma. RD1/ESX-1-deficient *M. marinum* strains are still able to recruit macrophages to the infection site, but fail to elicit aggregation into granulomas and intercellular bacterial spread and thus presumably lack the ability to induce chemotactic signals required for the initiation of these processes (Volkman et al., 2004). Primary granulomas can seed new granulomas by the efflux of infected macrophages, constituting a means of disseminating infection (Davis and Ramakrishnan, 2009).

The Role of Neutrophils

Macrophages have been described as the predominant cell type phagocytosing *M. marinum* after microinjection into fluid-filled compartments, such as the blood or hindbrain ventricle. In contrast, *M. marinum* seems to evade contact with neutrophils at initial infection sites. Instead, neutrophils are subsequently recruited to the nascent granuloma in response to signals from dying infected macrophages within the granuloma. Neutrophils are able to phagocytose and rapidly kill the internalized mycobacteria through NADPH oxidase-dependent mechanisms (Yang et al., 2012). Interestingly, effective uptake of *M. marinum* by neutrophils has been observed after subcutaneous infection of zebrafish (Colucci-Guyon et al., 2011). Likewise, it was found in the zebrafish model of *Escherichia coli* infection that neutrophils efficiently engulf bacteria on tissue surfaces, but not in fluid environments (Le Guyader et al., 2008). The concept that the route of entry of the bacteria into the host may determine the role of neutrophils in infections may also be of relevance for human infectious diseases, particularly for the early phase of encounter with microbes (Colucci-Guyon et al., 2011).

From Initial Granuloma Formation to Mature Granulomas

The view that granulomas are primarily host-beneficial protective structures, is thus challenged by the described findings in the zebrafish embryo model of *M. marinum* infection. Results demonstrate that pathogenic mycobacteria have developed mechanisms to harness nascent host granulomas for their dissemination and proliferation (Ramakrishnan, 2013). Pathologically, granulomas are defined as organized collection of differentiated macrophages with characteristic morphology, such as epithelioid histiocytes and giant cells (**Figure 2A**). In addition to macrophages, mature tuberculous granulomas in humans are populated by many other cell types, including neutrophils, dendritic cells, B and T cells, natural killer cells, fibroblasts, and epithelial cells (**Figure 2B**). The role of these cells in granulomatous infectious diseases is yet to be fully elucidated. Another characteristic of certain tuberculous granulomas is the presence of regions of acellular debris referred to as caseous necrosis. This limited central necrosis is also found in *M. marinum* infection; mycobacteria can be located both within macrophages and within the central caseous region. Microbial virulence factors may influence the cellular composition of granulomas and the role of these structures played in either the containment, persistence, or dissemination of infections.

INNATE IMMUNE MECHANISMS IN *M. ulcerans* INFECTION

Entry Point: Skin

The site of *M. ulcerans* and *M. marinum* lesions is thought to also be the site of inoculation of the mycobacteria into the skin, as indicated by the vast majority of patients presenting with a single skin lesion on body parts that are likely exposed to the contaminated environment and/or, in the case of *M. ulcerans,* to potential vectors (Portaels et al., 1999; Johnson et al., 2007; Lavender et al., 2011). Definitive evidence for this assumption is however lacking. For both mycobacterial skin infections, the skin epithelium constitutes the first physical barrier. In healthy skin, stable homeostasis and barrier function are established by resident keratinocytes, dendritic cells, T cells, mast cells, fibroblasts, and macrophages together with the resident microbiome (Gallo and Nakatsuji, 2011). Contact with environmental reservoirs of *M. ulcerans* or *M. marinum,* and penetration of the bacilli into the subcutaneous tissue through trauma – in the case of *M. ulcerans* potentially including bites by contaminated insects acting as mechanical vectors (Wallace et al., 2017) – may be the most common mechanism of infection, although other mechanisms cannot be excluded. The outcome of an infection with both *M. ulcerans* and *M. marinum* may depend on the mode of transmission and the initial dose of inoculated bacteria. In a guinea pig infection model, it has been shown that BU can be produced by intra-dermal injection, but not through inoculation of *M. ulcerans* onto a superficial abrasion (Williamson et al., 2014). In advanced BU lesions, clusters of extracellular AFB are predominantly found in deep layers of the subcutaneous fat tissue (Ruf et al., 2016). This may reflect the location of the initial inoculum causing disease or the presence of a microenvironment in the necrotic tissue which is most favorable for the multiplication of *M. ulcerans.* In a case series of patients with *M. marinum* infection, boating or fishing were associated with invasive disease, whereas fish tank exposure was associated with cutaneous disease. This may be related to the mechanism of injury; boating and fishing injuries may involve deep puncture wounds from fish spines, fishhooks or other equipment, while fish tank injuries may involve more superficial exposures such as minor scrapes while cleaning or maintaining fish tanks (Johnson and Stout, 2015).

Once Inside the Host. . .

Once inside the host, the mycobacteria encounter innate immune cells expressing various PRRs. Based on a series of *in vitro* experiments it was postulated that keratinocyte TLRs may play a role in the innate immune response to *M. ulcerans* infections (Lee et al., 2009). The precise role (if any) of keratinocytes in the recognition of *M. ulcerans* and subsequent modulation of innate responses in the host is however unknown. If we assume that the mode of transmission of the mycobacteria allows for an encounter with these cells, keratinocyte TLRs, in response to sensing PAMPs expressed by microbes and DAMPs produced by the host, can initiate an inflammatory cascade including the release of inflammatory cytokines and host antimicrobial molecules. Early events in the innate immune response to skin injury include the recruitment of neutrophils via chemokines released by the keratinocytes. Later during the inflammatory cascade, macrophages are the predominant immune cell type (Coates et al., 2018). At this stage of the infection, the ability to produce mycolactone results in a fundamentally

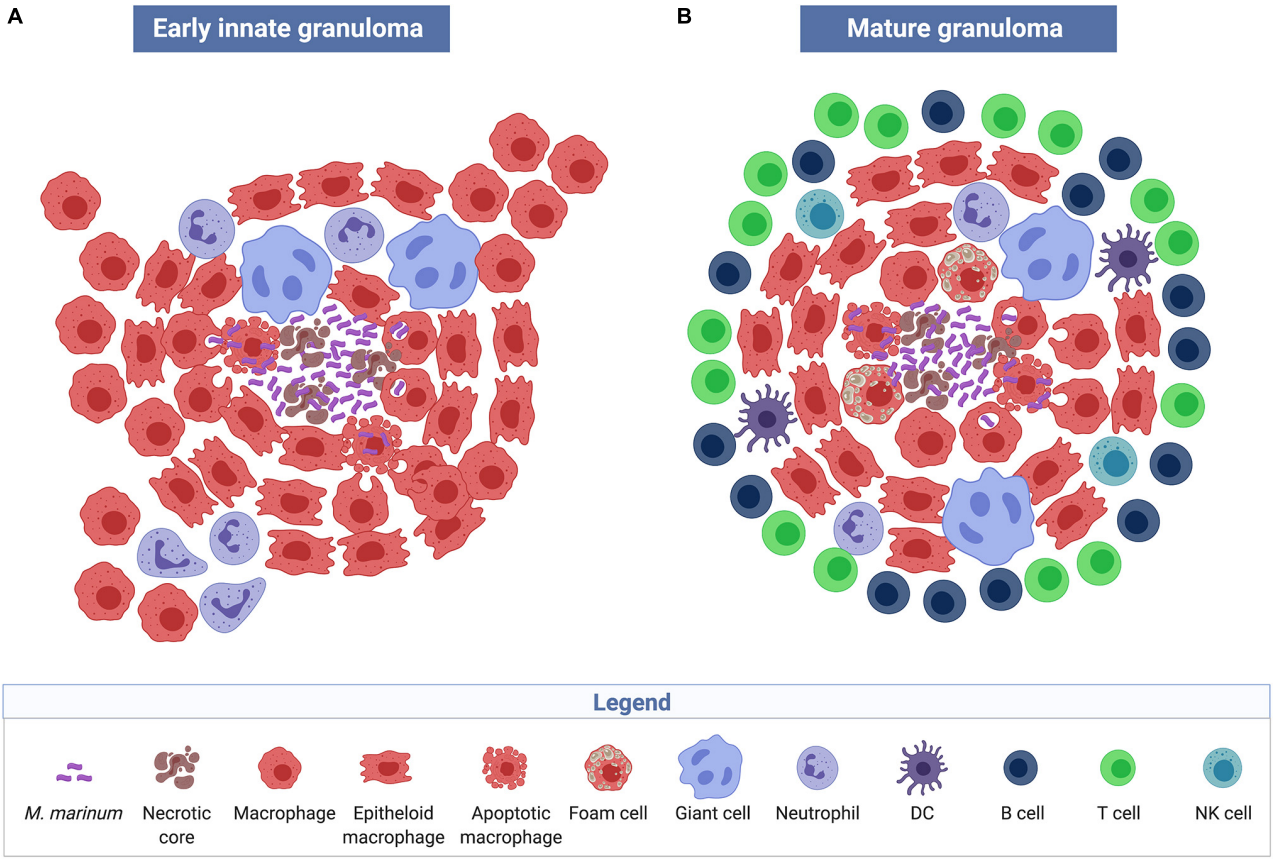

FIGURE 2 | Structure and cellular composition of granulomas. Early innate *M. marinum* granulomas, as described in zebrafish embryos, are composed of compact, organized aggregates of macrophages that have either transformed into epithelioid cells with tightly interdigitated cell membranes or have fused to become multinucleated giant cells. Bacilli are found both extracellularly in the necrotic core of the granuloma and intracellularly within phagocytes **(A)**. The characteristic mature tuberculous granuloma is populated by many other cell types such as neutrophils, dendritic cells, B and T cells, natural killer cells, and foam cells, which are macrophages with accumulated lipids **(B)** (created with biorender.com).

different interaction of the host with *M. ulcerans* as compared to *M. marinum*.

Insight Into Early Immune Defense Against *M. ulcerans* From Animal Models

To study immune responses setting in directly after infection with *M. ulcerans*, experimental infection models mostly involving mice (*Mus musculus*), but also other animals such as guinea pigs (*Cavia porcellus*) or pigs (*Sus scrofa*) have been developed (Bolz and Ruf, 2019). In the mouse model of BU, an immediate massive influx of neutrophils and to a lesser extent of monocytes/macrophages at the site of *M. ulcerans* intraperitoneal or intradermal injection is observed. One day after injection, bacilli are found within phagocytes and some of the inoculated bacilli are subsequently transported to the draining lymph nodes, where T_H1-type cellular immune responses are initiated. Prompted by these findings, an early intracellular growth phase of *M. ulcerans* that induces inflammatory cellular responses has been postulated (Coutanceau et al., 2005; Oliveira et al., 2005; Torrado et al., 2007; Silva et al., 2009). As early as 24 h after injection of *M. ulcerans* into mouse footpads, lysis of infected phagocytes mediated by the production of mycolactone by *M. ulcerans* causes release of the bacteria into the extracellular space (Oliveira et al., 2005). In particular globi-like accumulations of bacilli released from phagocytes (Schütte et al., 2009; Ruf et al., 2011) may readily form a protective cloud of mycolactone and may represent starting points for the development of large extracellular clusters. During this second stage of the infection, bacteria can multiply predominantly extracellularly, as mycolactone appears to prevent infiltrating immune cells from reaching the mycobacteria (Ruf et al., 2017). *M. ulcerans* forms an extracellular matrix, which is rich in proteins, lipids and lipoglycans and is likely to play a role in the development of extracellular clusters (Marsollier et al., 2007). High concentrations of mycolactone in the lesion core cause apoptosis of both resident skin cells and infiltrating leukocytes. Chronic, necrotic lesions develop upon the invasion of the bacteria into healthy tissue and the progressive lateral destruction of subcutaneous tissue (Oliveira et al., 2005). Ischemia associated with vascular pathology may also contribute to the coagulative necrosis. In the infiltrate surrounding the necrotic core, intracellular bacilli

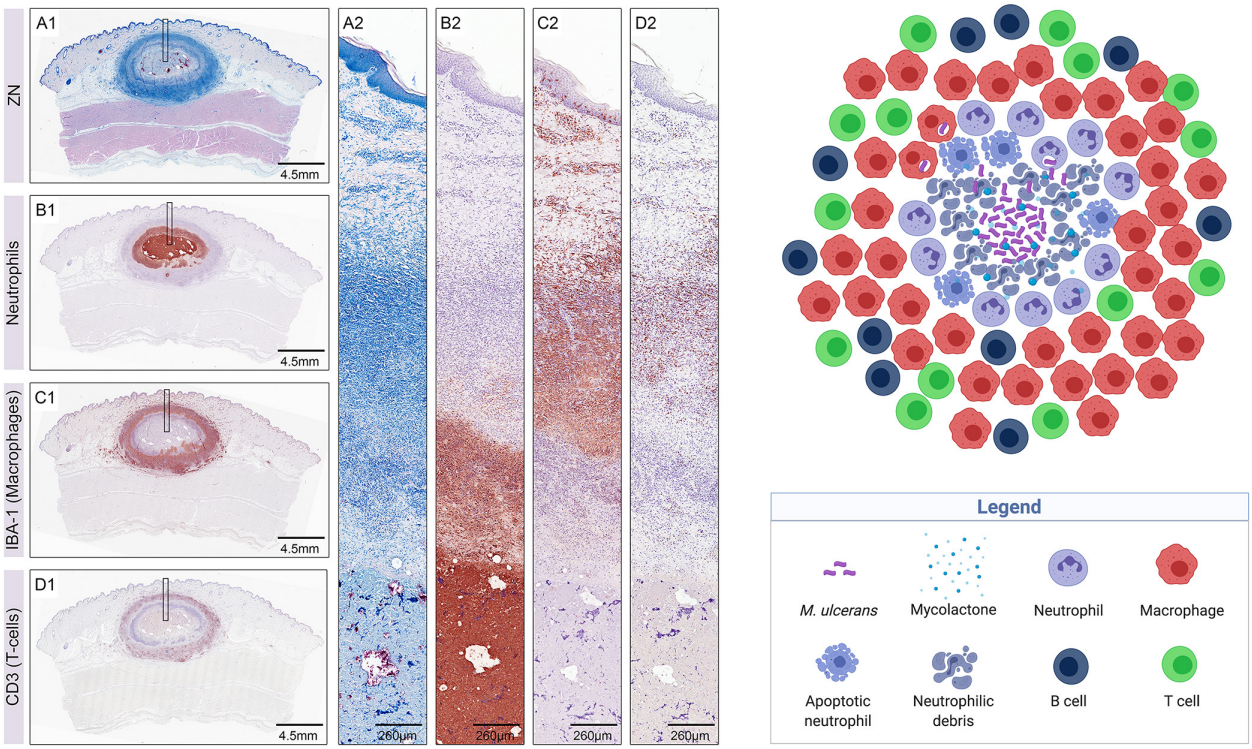

FIGURE 3 | Early host immune response to *M. ulcerans* infection illustrated in histological sections of a nodular lesion 6 weeks after subcutaneous infection of pigs **(A–D)** (from Bolz et al., 2016b) and in a sketch depicting the cellular composition of the lesion **(E)** (created with biorender.com). Histopathological analysis of pig lesions shows a central necrotic core containing extracellular *M. ulcerans* bacilli (**A1,A2**; AFB stained with Ziehl–Neelsen staining) and neutrophilic debris **(B1,B2)**. The necrotic core is surrounded by layers of neutrophils **(B1,B2)** and macrophages **(C1,C2)**, heavily interspersed with T-cells **(D1,D2)**.

can be detected, mainly in macrophages (Oliveira et al., 2005; Torrado et al., 2007). In the experimental mouse footpad infection model, dermal edema and footpad swelling become – dependent on the inoculation dose – evident a few weeks after infection. Necrosis extends to components of subcutaneous tissue, eventually resulting in extensive ulceration of the epidermis. That the continuous expansion of necrotic lesions is mainly mycolactone-mediated is illustrated in infections with mycolactone-negative *M. ulcerans* strains, which induce an initial acute neutrophilic response that gradually switches to a chronic mononuclear infiltrate devoid of massive necrosis (Oliveira et al., 2005).

The pig is widely used as a model in dermatological studies because pig and human skin share many morphological and physiological features. Pigs have been shown to develop single lesions at the site of injection of *M. ulcerans* into the skin, characterized by a central necrotic core containing large clumps of AFB surrounded by layers of neutrophilic debris, some intact neutrophils, and an outer belt of macrophages interspersed with T cells (**Figures 3A–E**). In contrast, lesions caused by mycolactone-deficient *M. ulcerans* strains present as multiple small central clusters of neutrophils and AFB with only limited necrosis, surrounded by a massive infiltration of macrophages interspersed with T-cells (Bolz et al., 2016b).

Interestingly, both wild type and mutant *M. ulcerans* strains evoke the same sequential infiltration layers with neutrophils and neutrophilic debris in the necrotic lesion centers surrounded by macrophages and T cells (Bolz et al., 2016b), a composition which is in contrast to granulomatous lesions caused by *M. marinum* or *M. tuberculosis*, characterized by organized aggregates of mature macrophages.

Insight Into Early Immune Responses From the Study of Early Human BU Lesions

Buruli ulcer patients in rural, endemic areas of Africa typically report to health facilities at late stages of the disease and thus histopathological studies are mostly restricted to advanced ulcers. In a cohort of 12 BU patients from far north Queensland (Australia) presenting with early lesions, immunohistochemical analysis revealed an acellular, necrotic core containing the extracellularly multiplying AFB separated from intact tissue by a belt of infiltrating immune cells comprising clusters of CD20-positive B cells, CD3-positive T cells, neutrophils, and macrophages. Neutrophilic debris was found inside the lesion core and is indicative of a massive early neutrophil infiltration that was walled off by the cytotoxic actions of mycolactone

(**Figure 3E**). Some AFB, located close to the infiltration belt, were detected intracellularly and may be signs of an expansion of the necrotic foci into healthy tissue (Ruf et al., 2017), a finding that was also reported in another study (Torrado et al., 2007). Although the pathogenesis of *M. ulcerans* infections in Australia and Africa seems comparable, it remains to be investigated if similar early immune responses are evoked in BU patients from Africa.

Immune Reconstitution After Antibiotic Therapy

Antibiotic treatment of BU patients results in a rapid onset of local cellular immune responses. Analysis of surgical specimens excised from patients after treatment with the standard antibiotic regimen for BU, revealed accumulation of infiltrating leukocytes in the BU lesions, presumably facilitated by decreasing concentrations of mycolactone associated with the suppression of the metabolic activity and finally with the killing of the bacilli. Already 4 weeks after start of antibiotic treatment AFB are primarily located within mononuclear phagocytes. Cellular infiltrates surrounding areas of coagulative necrosis display different levels of organization, including diffuse infiltrates present in all areas of connective and adipose tissue (**Figure 4A**) and, less frequently, organized epithelioid leukocyte accumulations located in deeper dermal tissue (**Figure 4B**) or dense lymphocyte aggregations in proximity to vessels (**Figure 4C**), reflecting a range of different functional activities required to clear the infection (Schütte et al., 2007).

Diverse Outcome of Infection

The early immune response may in many cases be capable of clearing an initial *M. ulcerans* inoculum. Spontaneous healing of BU lesions and serological evidence of exposure of healthy individuals to *M. ulcerans* indicate that the host immune system can contain infections with the pathogen, although mechanisms conferring protection are not entirely clear. Progression of lesions is highly diverse and not all non-ulcerative lesions ulcerate (Capela et al., 2015). One factor for the diverse outcome of *M. ulcerans* infections may be the inoculation dose. In the pig model of *M. ulcerans* infection, lower inoculation doses led to limited tissue destruction and eventually to the clearance of the bacteria (Bolz et al., 2014). Healing of BU lesions without specific treatment has been described for nodules, small ulcers, and long-standing ulcerative lesions (Revill et al., 1973; Marion et al., 2016a; O'Brien et al., 2019). How frequently BU lesions heal spontaneously is however difficult to assess, as many patients with early stages of the disease, that are usually indolent and non-systemic, may not report to health facilities. In a series of 545 BU patients diagnosed at a BU treatment center in Benin, 5% of the cases healed without specific treatment (Marion et al., 2016a). Whereas subcutaneous injection of *M. ulcerans* into BALB/c or C57BL/6 mice eventually leads to irreversible ulceration and tissue necrosis, ulcerative lesions developing after infection of FVB/N mice with *M. ulcerans* healed spontaneously despite persistent bacterial load. The healing process in FVB/N mice was associated with an infiltration of predominantly mononuclear cells, such as macrophages, dendritic cells, and neutrophils at the site of infection and an accumulation of myeloid cells in the draining lymph nodes, suggesting an important role for innate cellular immune defense mechanisms in protection (Marion et al., 2016b). In contrast to FVB/N mice, experimentally infected guinea pigs and pigs are able to entirely clear the *M. ulcerans* bacilli during the process of spontaneous healing (Bolz et al., 2014, 2016b; Silva-Gomes et al., 2015).

Children living in BU endemic areas of Africa seem to gradually develop resistance against BU with age, as indicated by a decline in the risk to develop the disease after a peak at an age of 12–14 years (Debacker et al., 2004, 2006; Bratschi et al., 2013). An increase in risk to develop BU in the elderly may be related to a deterioration of the relevant immune defense mechanisms. Prevalence of human immunodeficiency virus (HIV) infection in BU patients is significantly higher than in the local control population (Johnson et al., 2008; Christinet et al., 2014), indicating that T_H1 cell responses are important enhancers of the innate cellular immune defense against *M. ulcerans* infection. Moreover, skin lesions in BU-HIV co-infected patients tend to be more severe and more often multifocal (Johnson et al., 2002; Komenan et al., 2013). Case control studies aiming at the identification of host genetic factors relevant for susceptibility to BU have focused so far primarily on polymorphisms in genes known to be relevant for intracellular mycobacterial infections (Stienstra et al., 2006; Capela et al., 2016; Bibert et al., 2017). Significant associations observed with susceptibility to BU include polymorphisms affecting the promoter activity of the *IFN-γ* gene, the inducible nitric oxide synthase gene *iNOS* and the natural resistance-associated macrophage gene *SLC11A1* (*NRAMP1*). These results for the primarily extracellular *M. ulcerans* support the view that macrophages are crucially important for the early containment of *M. ulcerans* infections. Diversity of the response to human infection may thus be influenced not only by factors like inoculation dose, age, and nutritional status, but also by a complex constellation of genetic factors.

CONCLUSION

Despite common ancestry and the high degree of genetic relatedness of *M. marinum* and *M. ulcerans*, the role of the innate host immune system in immune defense against the two pathogens seems to differ considerably. Macrophages are a key component in the innate immune response to *M. marinum* infection, where they inadvertently play a dual role, both containing mycobacterial growth and providing an environment where the bacilli can persist (Ramakrishnan, 2012). Continuous recruitment of macrophages (and other immune cells), at least partly dependent on mechanisms of the RD1/ESX-1 secretion system, leads to a characteristic localized inflammatory response and granuloma formation. On the contrary, phagocytes seem to be only transiently inhabited by *M. ulcerans* in very early stages of the infection (Coutanceau et al., 2005). Histopathological analyses of human BU lesions and of those of experimentally infected animals indicate that neutrophils are the main infiltrating cell type in early stages of *M. ulcerans*

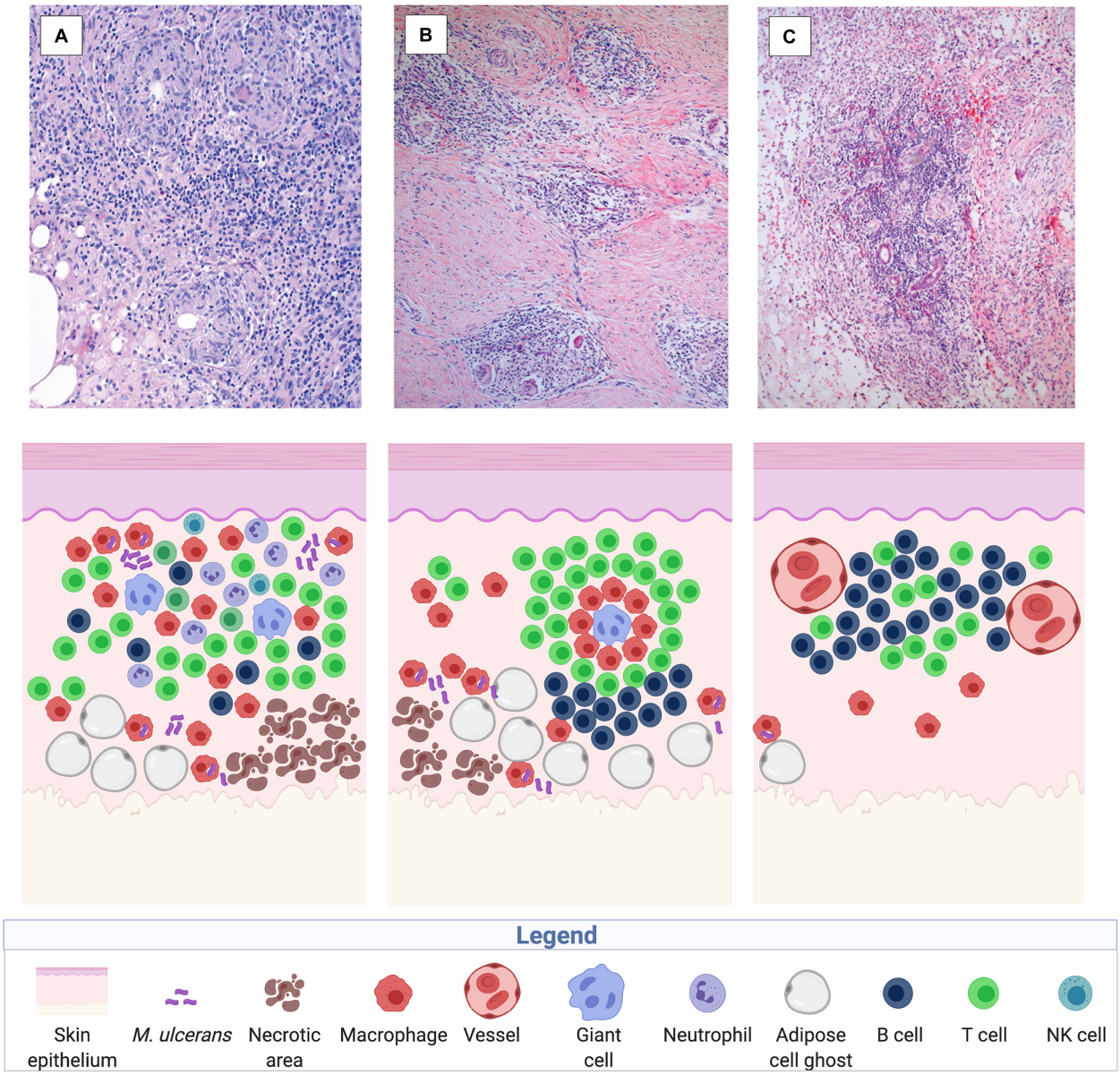

FIGURE 4 | Immune reconstitution responses after chemotherapy illustrated in histological sections of a human BU lesion (from Schütte et al., 2007) and in a sketch of the participating immune components (created with biorender.com). Three main types of cellular infiltration have been observed in BU patients after treatment with the standard antibiotic regimen, including diffuse, heterogeneous cellular infiltration of the connective and adipose tissue (100× magnified) **(A)**, granuloma-like structures in the connective tissue (40× magnified) **(B)**, and follicle-like lymphocyte structures adjacent to vessels (40× magnified) **(C)**.

infection, later complemented by macrophages (Bolz et al., 2016b; Ruf et al., 2017). The early dominance of neutrophils may be explained by the route of *M. ulcerans* infection (Le Guyader et al., 2008; Colucci-Guyon et al., 2011). After a postulated short intra-phagocyte stage of *M. ulcerans*, production of mycolactone enables a second, extracellular growth phase. Bacterial clusters are formed, which produce a protective cloud of mycolactone, walling off and killing infiltrating immune cells. A progressive invasion of healthy tissue and potentially of phagocytes at the periphery of the lesions leads to the characteristic chronic, necrotic course of the disease (Ruf et al., 2017). In BU patients under chemotherapy, killing of the bacteria and consequently the decrease in mycolactone concentrations allows for an immune reconstitution reaction (Schütte et al., 2007).

Due to relatively unspecific first signs of *M. ulcerans* infections, the often indolent course of the disease, and the limited access of affected populations to medical care, patients commonly seek treatment primarily in advanced disease stages, leaving patients in many cases with permanent disabilities. This and the highly focal occurrence of the disease makes vaccination

of inhabitants of endemic areas the desired solution for BU control, particularly because the limited understanding of risk factors for BU and reservoirs/vectors of *M. ulcerans* have so far hindered other preventive measures. Lack of knowledge on protective host immune responses has however also complicated the design of a vaccine for BU. Intriguingly, there is striking paucity of humoral immune responses upon infection with *M. ulcerans*, revealed after experimental *M. ulcerans* infection in the BU mouse model (Bieri et al., 2016; Bolz et al., 2016a), and corroborated by reports that sera of only a minority of BU patients contain *M. ulcerans*-specific antibodies (Yeboah-Manu et al., 2012). These findings may be explained by the cytotoxic and immunosuppressive actions of mycolactone on immune cells. This and evidence pointing toward an early intracellular phase of *M. ulcerans* call for a robust engagement of innate immune cells to boost cell-mediated immunity, eliminating the phagocytosed mycobacteria before larger intra- and later on extra-cellular toxin-producing bacterial clusters can form. Efficient activation of T cells orchestrated by antigen presenting cells is crucial to enable in turn activation (CD4 T cells) of host cells, such as macrophages to kill invading pathogens or direct killing (CD8 T cells) of infected host cells. The specific types of mycobacterial T cell antigens conferring protective immunity are yet to be determined. In addition to adaptive T cells activated upon mycobacterial peptide antigens presented by major histocompatibility complex (MHC) molecules, innate-like T cells with low antigen receptor diversity, which recognize lipid antigens presented by cluster of differentiation 1 (CD1), have been described (Beckman et al., 1994). T cells recognizing mycobacterial glycolipid antigens, which share some biological properties with both adaptive and innate-like T cells were shown to confer protection to tuberculosis in animal models (Larrouy-Maumus et al., 2017; James and Seshadri, 2020). In the past, strategies for the development of vaccines have mainly been empirical, with limited understanding of the underlying immune mechanisms, using killed or live attenuated forms of the pathogens. Live-attenuated vaccines such as BCG in the case of *M. tuberculosis* seem to be suitable for intracellular pathogens as they induce a broad range of immune responses including strong CD8 T cell responses. Interestingly, it has recently been reported that a prolonged increase in antimicrobial function of innate immune cells can itself contribute to protection from reinfection. In this context it was shown that BCG is capable of inducing non-specific cross-protection against microbes, a phenomenon that has been associated with a memory-like response in innate immune cells (Netea et al., 2011, 2016; Koeken et al., 2019). Such "trained innate immune cells" display functional and epigenetic reprogramming, leading to increased production of cytokines and chemokines, and improved phagocytotic and killing activities. Indeed, BCG was shown to offer a short-lived protective effect against BU in the first year after vaccination but limited to no protection thereafter (The Uganda Buruli Group, 1969; Smith et al., 1976). Whether this observed effect is due to mechanisms of trained immunity or is based on the broad antigenic cross-reactivity between *M. bovis* and *M. ulcerans* is however unknown. Similar results were found in the mouse model of experimental *M. ulcerans* infection, where BCG was shown to induce an immune response transiently containing proliferation of the bacilli but ultimately failing to prevent the typical BU pathology (Fraga et al., 2012). Differences in the effectiveness of BCG vaccination in different mouse strains has been reported (Converse et al., 2011). Genetically engineered BCG developed as a vehicle for BU vaccines offered marginally improved protection in the mouse model of *M. ulcerans* infection (Hart et al., 2015; Hart and Lee, 2016).

Considering that successful toxoid vaccines such as those against diphtheria and tetanus exist, targeting mycolactone itself may be a promising approach for the development of a BU vaccine. The presence of pre-existing neutralizing antibodies against mycolactone may physically block toxin interactions with host cells and thus aid cellular immune responses to the pathogen. Immunization of mice with a carrier protein conjugate of a non-toxic, synthetic mycolactone derivative has enabled for the first time the generation of antibody responses against the poorly immunogenic, cytotoxic mycolactone. Intriguingly, mycolactone-specific immune sera and mycolactone-specific mouse monoclonal antibodies showed toxin neutralizing activity, preventing mammalian cell apoptosis in an *in vitro* assay (Dangy et al., 2016). A more holistic approach targeting protective immune responses against both mycolactone and against other protein and potentially also glycolipid antigens may improve protective efficacy. To increase immunogenicity by activating appropriate elements of the innate immune system such a vaccine may need to be formulated with adjuvants stimulating various PRRs on innate immune cells (Tima et al., 2016).

Better understanding of the exact innate and adaptive immune mechanisms leading to protection from BU will help in the development of new strategies for effective vaccine design.

AUTHOR CONTRIBUTIONS

GP was invited to writing the review. KR and GP drafted and revised the manuscript.

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REVIEW
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Innate Immune Sensing by Cells of the Adaptive Immune System

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Sensing of microbes or of danger signals has mainly been attributed to myeloid innate immune cells. However, T and B cells also express functional pattern recognition receptors (PRRs). In these cells, PRRs mediate signaling cascades that result in different functions depending on the cell's activation and/or differentiation status, on the environment, and on the ligand/agonist. Some of these functions are beneficial for the host; however, some are detrimental and are exploited by pathogens to establish persistent infections. In this review, we summarize the available literature on innate immune sensing by cells of the adaptive immune system and discuss possible implications for chronic infections.

Keywords: TLRs, PRRs, DAMPs, B and T cells, adaptive immunity, chronic infections

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INTRODUCTION

For many years, sensing of conserved structures called pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPS) was thought to be a prerogative of myeloid cells of the innate immune system, such as macrophages, dendritic cells, or neutrophils. In the context of infectious diseases, innate immune sensing is responsible for launching a potent initial inflammatory response aimed to non-specifically eliminate invading pathogens. This initial defense mechanism is typically followed by a more specific and targeted response, which is orchestrated by cells of the adaptive immunity, namely B and T lymphocytes. It is owed to this notion that PAMP and DAMP sensing is extensively researched in innate immune cells, whereas innate immune sensing in lymphocytes has only recently been demonstrated and literature on physiological and pathological implications is still sparse. These mechanisms have perhaps been previously overlooked because lymphocyte activation typically occurs at later stages of infection, when inflammation is already established; however, recent evidence suggests that innate immune signaling can not only participate in lymphocyte maturation and improvement of B and T cell responses, but also be hijacked by pathogens such as *Leishmania donovani* to exacerbate detrimental immunosuppressive effects and induce hypergammaglobulinemia.

For the purpose of this short review, we will discuss the emerging field of innate immune sensing by cells of the adaptive immunity and its implications in *Leishmania* and other neglected tropical diseases such as Trypanosomiasis in two parts, placing individual focus on the two major actors of adaptive immunity, B and T lymphocytes.

INNATE IMMUNE SENSING IN B CELLS

Expression and Functions of Innate Immune Sensors in B Cells

Toll-Like Receptors (TLR) were the first innate immune sensors to be attributed a role in B cells. TLRs are a family lectin-rich repeats containing transmembrane proteins located on the cell surface (TLR1, TLR2, TLR4-6, and TLR10-11) or inside

the endosome (TLR3, TLR7-9). Murine B cells express TLR1-4, TLR6, TLR7, and TLR9 at varying levels in different subsets (1), while TLR expression on human B cells includes TLR1, TLR2, TLR6, TLR7, TLR9, and TLR10 (2), and in the case of plasma cells also TLR3 and TLR4 (3). Several beneficial roles of TLR-mediated sensing in B cell have been demonstrated, including the promotion of B cell maturation through TLR4 stimulation (4, 5) and enhanced antigen presentation by TLR9 ligation (6). While TLR engagement has been proposed to act as an additional signal to B cell receptor (BCR) stimulation (7), B cells have been shown to produce various cytokines and chemokines solely from TLR triggering (8).

Recent discoveries of cytosolic innate immune sensing pathways, including sensing of cytosolic DNA involving the adaptor protein stimulator of interferon genes (STING, also termed MYPS, MITA, ERIS) or cytosolic RNA via the adaptor MAVS have greatly advanced our understanding of immunity. A variety of sensor proteins, such as cyclic GMP-AMP synthase (cGAS) and interferon-induced protein 16 (IFI16), have been proposed to directly interact with DNA, leading to enzymatic generation of a secondary messenger molecule in the form of cyclic dinucleotides (CDNs), such as 2′3′-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP). These CDNs can then activate STING on surface of the endoplasmic reticulum to interact with TANK-binding kinase 1 (TBK1), resulting in phosphorylation of interferon regulatory factor 3 and subsequent IFN-I production (9).

Expression and functionality of STING and its pathway have also been demonstrated in B cells, although there is a discrepancy between cells of human and murine origin. Reports unanimously confirm STING expression in murine B cells, and have demonstrated that B cells are capable of responding to STING stimulation by production of IFN-I (10, 11); however, conflicting literature exists on STING expression and function in human B cells. In one study on peripheral blood mononuclear cell (PBMC)-derived human B cells, the presence of STING was confirmed via both flow cytometry and qPCR (12), while another study failed to detect STING via RT-qPCR in primary B cells from tonsils and PBMCs, but confirmed the expression of upstream (cGAS, IFI16) and downstream (TBK1, IRF-3) signaling partners (13). One possible explanation for the observed differences could be differences in the EBV-status of donors, as STING has been shown to be expressed in EBV-positive B cell lines, but not in EBV-negative cell lines. Both aforementioned studies did not observe IFN-I production from human B cells upon transfection of dsDNA or its synthetic homologs, which could be due to the low transfection efficacy into B cells or point toward an intrinsic defect of the STING signaling pathway in human B cells; however, Dong et al. observed a negative regulatory role of STING signaling in B cells on the JAK1-STAT1 pathway, suggesting a functional role of STING in B cells (12). STING activation has also been shown to upregulate costimulatory molecules, such as CD86, across all B cell subsets, have adjuvant activity following immunization with thymus-dependent antigens, improving antigen-specific antibody responses, and mediate apoptosis both in normal and malignant B cells (14, 15).

Another important nucleic acid sensing pathway in the cytosol is the pathway involving mitochondria antiviral-signaling protein (MAVS, also termed VISA, IPS-1, or Cardif). Several proteins have been suggested to act as cytosolic RNA sensors, including retinoic-inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5). These sensors can then cause aggregation of MAVS, leading to the activation of IRF-3 and IRF-7, NF-κB and production of IFN-I (16).

Both MAVS and its upstream sensors, RIG-I and MDA-5, have been shown to be expressed in B cells of human and murine origin, and were demonstrated to have a functional sensing pathway. Stimulation of B cells using the synthetic RNA analog poly(I:C) was shown to induce cytokines, predominantly IFN-β and IL-6, and to a lower degree IFN-γ, in a MAVS-dependent manner (13, 17). Additionally, triggering of the RIG-I/MAVS pathway using 5′-ppp-RNA was shown to be an effective adjuvant in influenza vaccination, leading to a long-lasting antibody response of improved specificity (18).

Pathological Implications of Innate Immune Sensing in B Cells

The identification of a role of innate immune sensing in the cytokine and antibody production in B cells attracted considerable attention in the field of autoimmunity research. Multiple studies have confirmed the involvement of TLR signaling, in particular MyD88-dependent TLRs and endosomal TLR7 and TLR9, in autoreactive B cell activation and germinal center (GC) formation (19), autoantibody production (20, 21), and development of autoantibody-related pathologies such as glomerulonephritis in the context of the human disease systemic lupus erythematosus (SLE) (22, 23) and in models using the lupus-prone mouse strain MRL/lpr (24).

In a mouse model of the IFN-related autoimmune condition Aicardi-Goutières Syndrome (AGS), which is mimicked by a deletion of dsDNA-degrading protein 3′ repair endonuclease 1 (Trex1), B cells were shown to be responsible for the development of glomerulonephritis and greatly contributed to disease-related mortality (25). A different study on lupus demonstrated a negative regulatory effect of STING on JAK1-STAT1 activation and found decreased STING expression in B cells from SLE patients and MRL/lpr lupus-prone mice (12).

MAVS, on the other hand, seems to be involved in regulation of germinal center formation. The formation of spontaneous germinal centers (Spt-GCs), whose dysregulation is associated with SLE and other autoimmune diseases, was shown to be dependent on MAVS and TLR7 expression in mice, and TLR7 ligation could only partially reinstate the Spt-GC development (26). Another study not only confirmed that MAVS in B cells is required for the formation of autoreactive GCs and autoantibody production in lupus-susceptible mice, but additionally linked its expression to the development of proteinuria and glomerulonephritis (27).

Signaling through pattern recognition receptors (PRR) in B cells was also reported to dysregulate processes leading to antibody production. Recent evidence suggests that innate immune activation might directly contribute to detrimental

antibody production, as increased TLR7 signaling has been observed to favor differentiation of lupus-associated $CD27^{-}IgD^{-}$ B cells into plasma cells excreting autoreactive antibodies, although co-stimulation by IL-21 and IFN- γ along with TLR7 is required to differentiate naïve B cells into these double negative and plasma cells. Furthermore, B cells from SLE patients were found to have increased expression of genes involved in innate RNA sensing, including TLR7, TBK1, and TRIM56, an inducer of STING (28). This is consistent with observations of a prominent IFN-I signature in SLE and other autoimmune diseases, which was shown to further upregulate TLR7 and TLR9 expression, thereby potentially amplifying detrimental autoantibody production (29).

Polyclonal B cell activation and subsequent excessive generation of antibodies, termed hypergammaglobulinemia, is not only a common feature of many autoimmune diseases in humans and in mouse models (30, 31), but is also a hallmark of many chronic infections, including leishmaniasis and Chagas disease (32, 33). As pronounced IFN-I production has been observed in models using *L. donovani* and *T. cruzi* (34, 35), similar mechanisms might be at play to exacerbate B cell activation and cause hypergammaglobulinemia in these diseases.

Not only do autoimmune diseases and chronic inflammatory diseases share many characteristics, including aberrant B cell activation and antibody production, but many pathogens have also been linked to the induction of autoimmune reactions. One of these pathogens is the intracellular protozoan parasite *Trypanosoma cruzi* which induces chronic chagasic cardiomyopathy (CCC) in 30–50% of patients and accompanied by high production of inflammatory cytokines, including IL-1β, IFN-γ, and TNF (36). Different roles for TLRs in *T. cruzi* infection have been proposed—lack of signaling through TLR7 and TLR9 has been found to enhance susceptibility to infection and decrease parasite clearance (37, 38), while TLR2 and TLR4 ligation were shown to modulate the pro-inflammatory response in the cardiac form, and in the anti-inflammatory response in the asymptomatic form of the disease (39); however, little information exists on the contribution of B cell-intrinsic TLRs in this context. Distinct clinical forms of Chagas' disease were found to have different underlying TLR expression and subsequent cytokine production in PBMCs. Elevated levels of TLR2 expression and concomitant production of pro-inflammatory cytokines TNF and IL-12 were found in patients exhibiting cardiac pathologies, while increased TLR8 and IFN-β expression was determined in the digestive forms (40). The frequency of TNF-producing B1 cells in cardiac patients was shown to be higher than in non-infected individuals and was significantly increased upon further exposure to *T. cruzi*-derived protein-enriched fraction (41); however, direct studies on B cells in *T. cruzi* infection are required to elucidate the contribution of B cell TLR signaling to this cytokine production.

Like trypanosomiasis, leishmaniasis is induced by a family of protozoan parasites belonging to the group of Trypanosomatids. Contrasting roles for B cells for different parasite strains have been demonstrated both in disease protection or progression dependent on the model organism and parasite strain [reviewed in (42)]; however, little is known about the contribution of innate immune sensing in B cells in the context of this disease. Using an experimental model of visceral leishmaniasis, our laboratory has previously demonstrated that *Leishmania donovani* amastigotes can induce production of pro-inflammatory cytokines, IFN-I, and IL-10, by engaging endosomal TLR3, TLR7, and TLR9. As *in vitro* exposure of B cells to the parasite also resulted in an IFNAR-dependent upregulation of endosomal TLR mRNA, we proposed a positive regulatory loop of IFN-I on endosomal TLR expression, thereby enhancing the modulatory effect of endosomal TLR signaling on cytokine production and antibody production, which results in hypergammaglobulinemia and disease exacerbation (34, 43). A similar feedback mechanism for IFN-β on TLR7 and TLR3 has been suggested by other groups, and investigation of the source of this IFN-I provides a link between the innate RIG-I/MAVS and TLR signaling pathways (17, 44): Loetsch et al. have found stimulation of the RIG-I/MAVS pathway with synthetic RNA analog to cause upregulation of endosomal TLR expression, namely TLR3 and TLR7, in an interferon-α/β receptor (IFNAR)- and partially MAVS-dependent manner. Thus, IFN-I produced via MAVS-mediated sensing pathways could partially account for the amplification of B cell activation and hypergammaglobulinemia through upregulation of endosomal TLRs; however, the reduction of TLR upregulation in the MAVS-compromised B cells does not fully account for the reduction observed in the *Ifnar*$^{-/-}$ mice. This suggests that there might be an additional source of type-I interferon produced by a MAVS-independent pathway. In fact, DNA derived from *L. donovani* has recently been demonstrated to be able to induce IFN-I production via the cGAS/STING pathway in macrophages (45), and while only the B-1 lineage of B cells has been demonstrated to be able to phagocytose *Leishmania* parasites (46), it is possible that parasite DNA is delivered to the B cell cytosol through yet unidentified pathways to trigger IFN-I production via cGAS/STING.

Finally, while innate immune signaling can thus be subverted by pathogens to exacerbate disease by dysregulating germinal center formation, antibody and cytokine production, targeted engagement of individual sensors, especially of the TLR family, has also been proposed in therapy of various inflammatory and infectious diseases (47); however, the effect of using TLR ligands as adjuvants in therapy can differ widely even in the same model, as demonstrated by a study on established cutaneous leishmaniasis infection caused by *L. (Vianna) panamensis*. TLR9 stimulation using high doses of its ligand CpG was shown to decrease lesion size, drastically reduced parasite burden, and decreased B cell-mediated IFN-γ, while stimulation with low doses of CpG increased IFN-γ production in the same cells (48), highlighting the importance of further studies on innate immune signaling and its effect on adaptive immunity in B cells in order to develop safe and effective treatments for chronic infectious diseases.

The low number of studies on cytosolic nucleic acid sensors in B cells in infectious diseases limits conclusions to be drawn for its relevance in pathological settings at this point in time. The use of a conventional knockout of cGAS in mice was shown to induce dysregulated germinal center and antibody responses and reduced parasite clearance in a non-lethal malaria

model; however the effect on GC formation was found to be B cell-extrinsic (49). Similarly, while MAVS^{-} mice infected with the non-pathogenic West Nile Virus-Madagascar (WNV-MAD) strain exhibited increased GC formation, antibody-titers and plasma cell formation, this effect was found to be dependent on MAVS signaling in dendritic cells rather than B cells (50). While both studies chose to focus on the most prominent role of B cells, production of antibodies, neither of them investigated the effect of cytosolic nucleic acid sensing on cytokine production. In B cells isolated from lungs and spleen of *Mycobacterium tuberculosis*-infected mice, a dramatic STING-dependent upregulation of IFN-β mRNA, along with a milder increase in IL-6 and a tendency toward upregulated IL-10 was observed. This marked increase of IFN-β production was also shown to be present in B cells purified from pleural fluid of Mtb patients as compared to healthy donors. Interestingly, a lower amount of IFN-β could also be triggered by poly(I:C), which is a ligand of TLR3 but can also be sensed via the RIG-I/MAVS pathway. Along with another interesting finding that MyD88 signaling suppressed STING-mediated IFN-β expression, this not only provides further evidence of functional cytosolic sensing in B cells in infectious diseases but also draws another connection between cytosolic and TLR sensing (51). Collectively, this implies that, while cytosolic sensing in B cells might have a limited direct effect on GC formation and antibody response, it can have substantial effect on modulating cytokine production, and, through interconnections between the different innate immune sensing pathways, potentially mediated through IFN-I, a central player in many of these pathways, indirectly mediate B cell responses.

Despite studies demonstrating the functionality of intracellular innate immune sensing pathways in B cells, the question still remains as to how these mostly non-phagocytic immune cells are able to recognize pathogen-derived nucleic acids in their cytosol. One possibility has been demonstrated in the case of *Listeria monocytogenes*, which can induce

TABLE 1 | Innate immune sensing in B cells.

PRR/adaptor	Function	Model/agonist	Organism
TLR2	↓ B cell maturation (5)	Pam3Cys	Mouse
	↑ Cytokine and chemokine production (8)	Pam3CSK	Human
TLR3	↑ Pro-inflammatory cytokines (34)	*Leishmania donovani*	Mouse
TLR4	↑ B cell maturation (4, 5)	LPS	Mouse
TLR7	↑ Cytokine and chemokine production (8)	Imiquimod	Human
	↑ Spontaneous GC formation (19, 26)	Imiquimod	Mouse
	↑ Autoantibody production (20)	Lupus-prone mice	
	↑ Pro-inflammatory cytokines, ↑ hypergammaglobulinemia (34)	*Leishmania donovani*	
TLR9	↑ Proliferation, ↑ survival, ↑ costimulatory molecule expression, ↑ antigen presentation (6), ↑ sensitivity to BCR stimulation (7), ↑ cytokine and chemokine production (8)	CpG (6, 7), GpG-ODN2006 (8)	Human
	↓ Spontaneous GC formation (19)	TLR9 knockout	Mouse
	↑ Autoantibody production (20)	Lupus-prone mice	
	↑ Pro-inflammatory cytokines, ↑ hypergammaglobulinemia (34)	*Leishmania donovani*	
	At high CpG doses (> 1 μM): ↓ lesion size, ↓ parasite burden, ↓ IFN-γ at low CpG doses (>40 nM) ↑ IFN-γ	CpG treatment in *Leishmania (Vianna) panamensis*	
cGAS	↓ Parasite burden, ↓ GC response (49)	*Plasmodium yoelii*	Mouse
STING	↑ Disease-related mortality, ↑ glomerulonephritis (25)	Aicardi-Goutières Syndrome	Mouse
	↓ JAK-STAT1 activation, ↓ antibody response (12)	Systemic lupus erythematosus (SLE)	Mouse/human
	↑ Cytokine production (51)	*Mycobacterium tuberculosis*	
MAVS	↑ Spontaneous GC formation (26), ↑ autoreactive GC formation, ↑ autoantibody production, ↑ disease-related pathology (27)	Lupus-prone mice	Mouse
	↑ TLR3 and TLR7 expression (17), ↑ cytokine production (51)	Poly(I:C)	

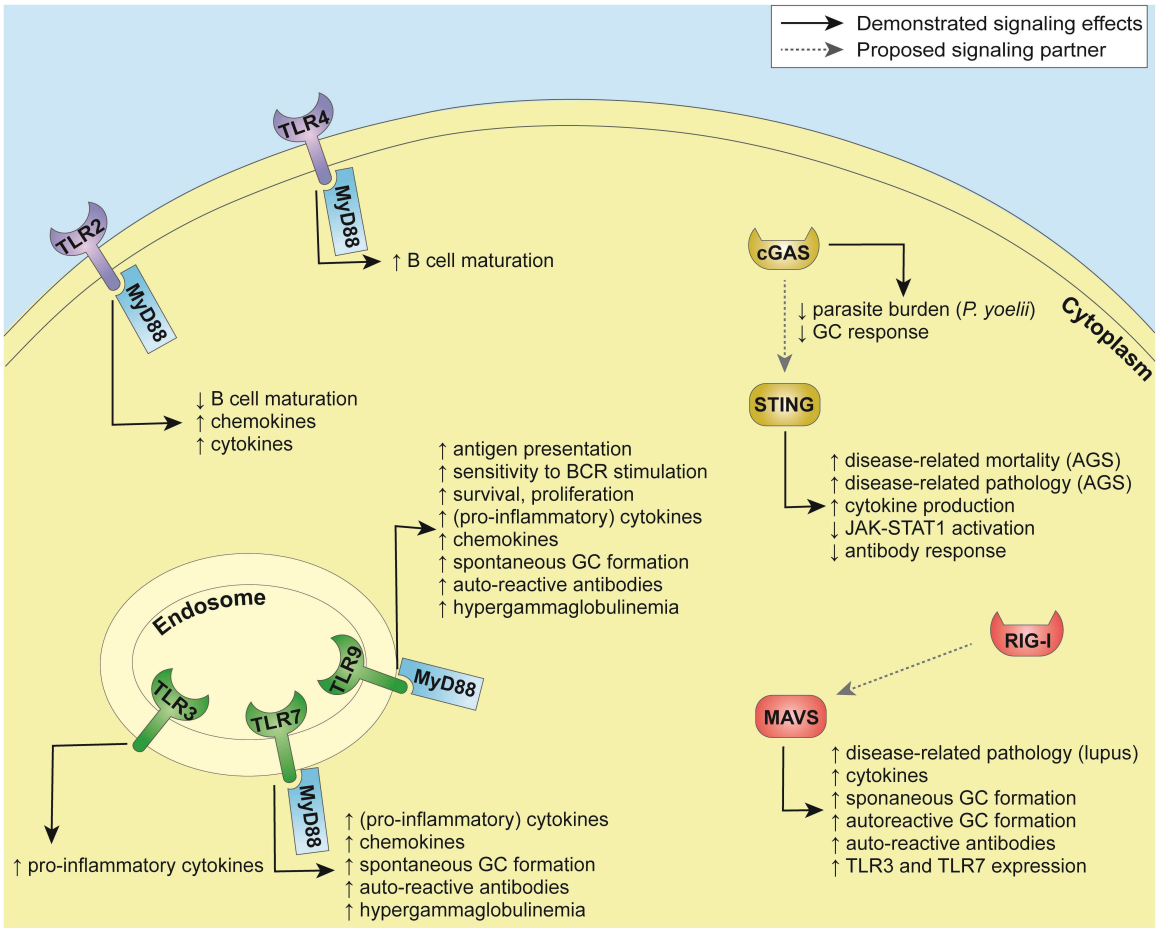

FIGURE 1 | Summary of pathways involved in innate immune sensing in B cells.

nucleotide sensing in non-phagocytic cells by secretion of bacteria-derived nucleic acids (52, 53). As many chronic infections are characterized by increased apoptosis and tissue disruption, as the case during hepatosplenomegaly in visceral leishmaniasis or cardiac damage in Chagas' disease (54, 55), it is also possible that this may promote release of host nucleic acids into the tissue environment (56), which could in turn induce innate immune sensors in surrounding cells, including B cells. Nevertheless, further investigations are warranted to identify the mechanisms underlying activation of intracellular sensors in B cells by pathogens.

Functional outcomes of PRR triggering in B cells are summarized in **Table 1** and **Figure 1**.

INNATE IMMUNE SENSING IN T CELLS

Expression and Functions of Innate Immune Sensors in T Cells

T lymphocytes have also been reported to express several PRRs; however, the downstream effect of PRR activation varies depending on T cell population, activation status, ligand, and/or environment.

Most of the available literature on PRR expression in T lymphocytes investigates the role of TLRs in T cell differentiation and effector function. Murine and human T cells were shown to express mRNA and protein for most of the TLRs (57–64); however, their expression intensity depends on T cell subsets and activation status. Interestingly, TLR expression seems to be regulated by TCR-dependent activation; indeed, antigen-experienced T cells express higher TLR levels than naïve cells (58, 59, 65). During priming, TLR activation appears to function as a sort of costimulatory signal enhancing effector function, proliferation, cell survival, and cytokine production in murine and human CD4 T cells (61, 64, 66, 67). For example, signaling through TLR9 can induce NF-κB activation in CD4 T cells via the adaptor molecule MyD88, leading to the upregulation of anti-apoptotic molecules and increasing cell survival (58). Similar anti-apoptotic functions were ascribed to TLR2 in CD8 T cells (68). Moreover, signaling throughTLR9- MyD88 also promotes CD4 T cells proliferation by activating a PI3K/Akt-dependent pathway (67).

Expression of costimulatory molecules and cell trafficking are also promoted by TLR stimulation. For instance, CpG ODN (TLR9 agonist) induces expression of OX-40 and CD40L on CD4 T cells during priming; while treatment with LPS increases adhesion capacity and inhibits chemotaxis of human and murine T cells (69, 70).

The importance of intrinsic MyD88-dependent signals in promoting CD8 and CD4 T cell survival and initial proliferation was also demonstrated in *in vivo* studies in various models of infection, including *Toxoplasma gondii* and Lymphocytic choriomeningitis virus (LCMV) infections (71, 72).

Moreover, MyD88 and TLR signaling appears to be essential for the differentiation of Th17. cells. The vital role of MyD88 was shown in experimental models of colitis and experimental autoimmune encephalitis (EAE). In the colitis model, *Myd88*$^{-/-}$ CD4 T cells showed reduced survival, failed to induce severe disease, and poorly differentiated into Th17 cells (60, 73). Later studies in the EAE model suggested TLR2 and TLR4 signaling as being crucial for the differentiation of Th17 cells (64, 74, 75). Indeed, TLR2 activation in CD4 T cells seems to synergize with IL-23 to induce Th17 cells; additionally, TLR2-deficient CD4 T cells fail to induce EAE and to differentiate into IL-17 or IFN-γ-producing cells in adoptively transferred mice.

Taken together, the literature suggests that TLR signaling plays an important role in providing cell survival and proliferative signals during T cell priming and in enhancing effector functions and cell differentiation.

Some cytosolic nucleic acid sensors were also reported to be expressed in T cells. For instance STING expression was detected in human and murine T cells (76–78); RIG-I is expressed in human peripheral T lymphocytes (79); LGP2 is present in murine CD8 T cells (80); and the immune sensor NLRC3 was observed in murine CD4 T cells (81). With exception of LGP2, all other pathways appear to impair T cell proliferation, function, or survival. Hence, their role in T cells will be discussed in the next section. LGP2 is a member of the RIG-I-like receptors family of cytosolic RNA helicases that includes RIG-I and MDA5. Unlike RIG-I and MDA5, which are known to initiate the activation of IRF-3 and NF-κB to induce expression of IFN-I, LGP2 can function as a negative regulator of RLR signaling inhibiting TLR-independent sensing of viral replication (82) and RIG-I multimerization (83), or compete with MAVS to suppress innate immune signaling (84). A positive function for LGP2 as a cofactor for RLR signaling of RIG-I and MAVS-mediated antiviral responses has also been described, but the mechanism is yet unknown (85, 86). In CD8 T cells, LGP2 promotes cell fitness and survival by controlling sensitivity to death-receptor signaling during acute West Nile virus and LCMV infections. Indeed, LPG2-deficient CD8 T cells display enhanced activity of caspase 8, 3, and 7 and enhanced expression of death receptors TNFR-I, TRAILR2 (or DR5), and CD95 (or Fas receptor) (80).

Immunosuppressive Effects of Innate Immune Sensing in T Cells

Despite the strong evidence that T cell-intrinsic PRR activation complements TCR and costimulatory signals to improve T cell responses during priming, a few studies have reported an inhibitory role for innate immune sensing in T cells.

Signaling via TLR2, for instance, was shown to inhibit T cell chemotaxis through upregulation of the transcription factor SOCS3 (suppressor of cytokine signaling 3) (87). An additional study reported that TLR2 was also involved in downregulating the transcription factors T-bet and NF-κB (88). Both studies used Heat shock protein 60 (HSP60), arguably a DAMP, to stimulate T cells. In contrast, in CD25+ CD4+ regulatory T cells (Tregs) exposed to HSP60 upon activation with anti-CD3, TLR2 was required to enhance their immunosuppressive effects via activation of PCK, PI-3 kinase, and p38 (89). Stimulation of Tregs with pathogen-derived TLR2 ligands induced proliferation and promoted survival (65, 89–91); however, whether this enhances (89, 91) or curbs (65, 90) their inhibitory function is still controversial.

While TLR4 activation is essential to drive Th17 responses (75), its effects on Th1 cells are rather inhibitory in a spontaneous model of colitis (92) and in human T cells exposed to LPS (70), where signaling via TLR4 inhibited cell migration. Inhibitory effects were also ascribed to the TLR7 activation pathway in CD4 T cells. In a model of EAE, triggering of TLR7 suppressed Th17 cell differentiation, which resulted in reduced disease severity (93). This effect was mediated by downregulation of STAT3 and induction of SOCS3 and SOCS5 (93). Furthermore, in human T cells purified from the blood of HIV^{+} individuals, TLR7 stimulation promoted the activation of an NFATc2-dependent anergic gene-expression program, which led to cell unresponsiveness (62). Work from our laboratory has also revealed an inhibitory function for TLR7 activation in Th1 cells in *L. donovani* infected mice. During the chronic stage of disease, Th1 cells increasingly upregulated TLR7 expression and sensed DAMPs derived from inflammatory tissue disruption (59). Engagement of TLR7 on those cells resulted in the activation of the transcription factor interferon regulatory factor 5 (IRF-5), which induced the transcriptional activation of death receptor 5 (DR5 or TRAILR2) and caspase 8, thereby promoting cell death (59).

TLRs are not the only innate immune sensors capable of inhibiting T cell functions. The STING pathway was recently shown to be active in T cells as well. Stimulation with STING agonists not only promoted IFN-I production and interferon stimulated genes' expression, but it also led to the down-regulation of anti-apoptotic and the upregulation of pro-apoptotic genes (78). Interestingly, T cells exhibit an intensified STING pathway, which results in a different gene expression profile to innate myeloid cells and leads to cell death (94). Moreover, activation of STING was shown to have an antiproliferative effect in human and murine CD4 T cells. This antiproliferative capacity requires STING relocalization to the Golgi apparatus (76).

The RIG-I pathway seems to also have inhibitory effects in human T cells. Zhang et al. report a positive correlation between RIG-I expression in peripheral T cells and T lymphocyte counts in patients affected by dermatomyositis. Interestingly, RIG-I induced apoptosis in these cells and inhibited their proliferative capacity (79).

TABLE 2 | Innate immune sensing in T cells.

PRR/adaptor	Function	Model/agonist	Organism
MyD88	↑ Disease susceptibility (71)	*Toxoplasma gondii*	Mouse
	↑ Clonal expansion, ↑ survival (72)	Lymphocytic choriomeningitis virus (LCMV)	
	↑ Survival, ↑ disease induction, ↑ Th1 and Th17 differentiation (60, 73) ↑ Expression of anti-apoptotic molecules (73)	Colitis	
TLR2	↑ Anti-apoptotic molecules, ↑ Survival, ↑ proliferation (65, 68)	Pam(3)CysSK(4)	Mouse
	↑ Th17 differentiation, ↑ Th17 cytokine production (64)	Experimental autoimmune encephalomyelitis (EAE)	
	↑ Tregs proliferation, ↑ survival (65, 89–91) ↑ Inhibitory Treg function (89, 91) ↓ Inhibitory Treg function (65, 90)	Pam3CysSK4 (65), HSP90 (90), *Candida albicans* (91) Bacteroides fragilis (92)	
	↓ Chemotaxis (87), ↓ Th1 responses (88, 89)	HSP60	Human/mouse
TLR3	↑ Anti-apoptotic molecules, ↑ Survival (58)	Ligation with poly(I:C)	Mouse
TLR4/MyD88	↑ Adherence, ↑ chemotaxis (70)	Ligation with LPS	Human
TLR4	↑ Th17 differentiation, ↑ Th17 cytokine production (75)	EAE	Mouse
	↓ Th1 responses (92)	Colitis	
TLR7	↑ Th1 cell death (59)	*Leishmania donovani*	Mouse
	↓ Th17 differentiation (93)	EAE	
	↓ Proliferation, anergy (62)	HIV-1	Human
TLR9/MyD88	↑ Anti-apoptotic molecules, ↑ Survival (58), ↑ proliferation (67)	Ligation with CpG	Mouse
RIG-I	↓ Proliferation, ↑ apoptosis (79)	Dermatomyositis	Human
LPG2	↑ Survival, ↓ death receptor signaling (80)	RNA viruses	Mouse
STING	↓ Proliferation (76)	Activating STING mutations	Human/mouse
	↑ ER stress, ↑ apoptosis (78, 94)	DMXAA (78), CMA (95)	Mouse
NLRC3	↓ Th1 proliferation, ↓ Th17 proliferation, ↓ Cell metabolism (81)	LCMV, EAE	Mouse
Unknown nucleic acid sensor	↑ Co-stimulatory responses, ↑ Th2 differentiation (97)	Ligation with TLR ligands, synthetic NA analogs, self-DNA from dead cells	Mouse

Another molecule involved in innate immune sensing pathways that was recently shown to reduce T cell effector functions is NLRC3 (95). NLRC3 belongs to the group of non-inflammasome-forming NLRs (NOD-like receptors), together with NOD-1, NOD2, among others. NLRC3 is a known negative regulator of innate immunity and inflammatory responses (95, 96). This molecule is highly expressed in T cells, where it seems to fine-tune CD4 T cell activation by attenuating IFN-γ and TNF expression, decreasing proliferation of Th1 and Th17 cells, and affecting cell metabolism by reducing glycolysis and oxidative phosphorylation (81).

It was also demonstrated that T cells are capable of sensing nucleic acids via pathways distinct of those identified so far in the innate immune system. Interestingly, higher-order structure of the nucleic acids was required for their internalization by T cells; indeed, self-DNA released from dead cells and complexes with antimicrobial peptides or histones induced costimulatory responses upon recognition by yet unidentified sensor(s), promoting the differentiation into Th2 cells (97). Downstream effects of innate immune sensing in T cells are summarized in **Table 2** and **Figure 2**.

It is interesting to observe that innate sensing by T cells during the priming phase leads mainly to a positive outcome:

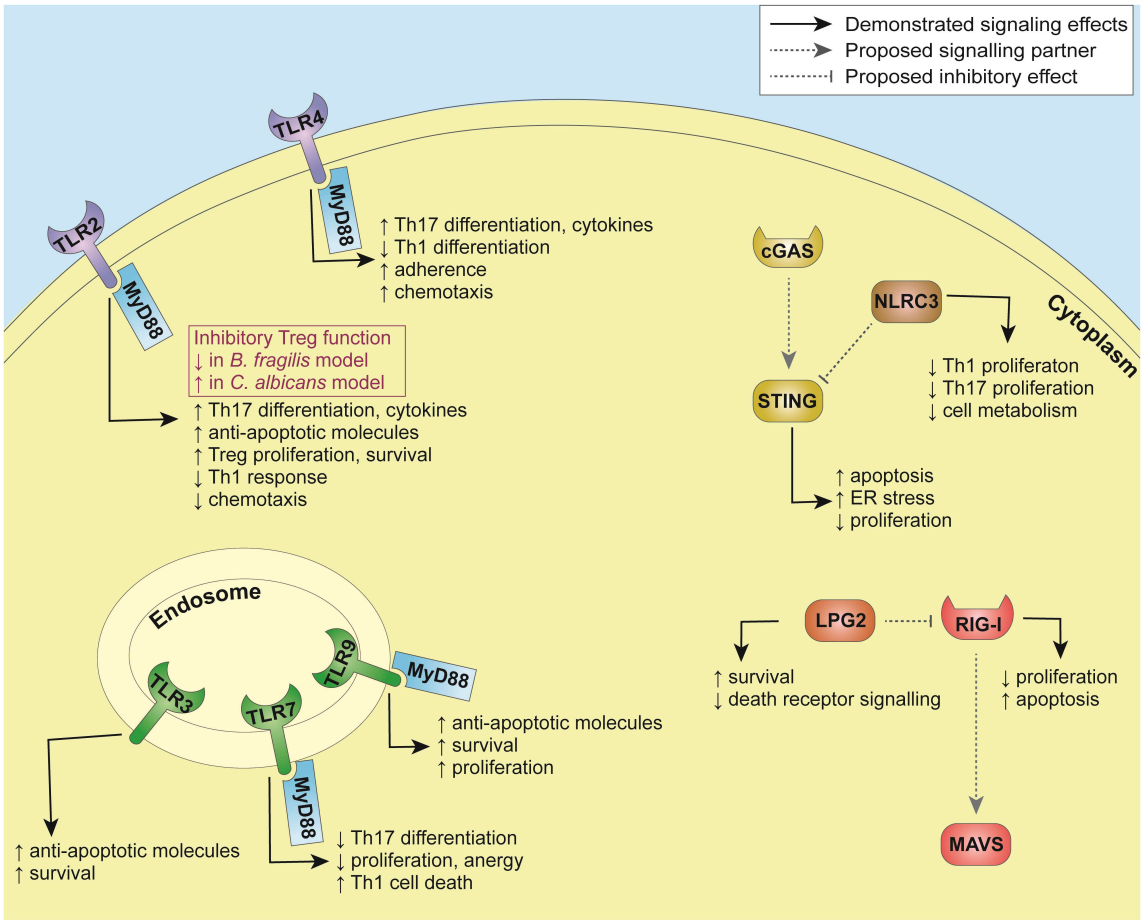

FIGURE 2 | Summary of pathways involved in innate immune sensing in T cells.

it promotes cell survival, enhances effector function, and helps differentiation. In contrast, during chronic infections or in a chronic inflammatory environment, PRR activation in T cells results in cell death and anergy, and limits the cells' proliferative capacity. It is yet unclear why PRR signaling mediates such disparate functions depending on the cell differentiation stage and the inflammatory environment. Further studies are definitely warranted to better define the level of expression, signaling pathways and downstream targets of PRRs in various T cell populations. Also, very little information is currently available on innate immune sensing by T cells through PRRs that are upregulated during chronic infection and the importance these may have in helping pathogen persistence and inhibiting protective T cell responses. Furthermore, the nature of the ligands responsible for triggering those responses in T cells, and the stimuli required for promoting PRR expression during chronic infections have also not yet been identified. We have demonstrated that DAMPs could trigger TLR7 and induce cell death in T cells isolated from *L. donovani*-infected mice during chronic infection (59). Inflammatory tissue damage is a common characteristic of persistent infections and release of several DAMPs through this process is inevitable. It is thus possible that other PRR, such as STING and RIG-I, are also activated by DAMPS derived from tissue damage during chronic visceral leishmaniasis. While curbing of pro-inflammatory T cell responses in a chronic inflammatory environment could represent a protective mechanism to prevent tissue disruption, it may also favor pathogen persistence. Indeed, in our model, disruption of the TLR7 signaling pathway in T cells resulted in stronger Th1 responses and a lower parasite burden (59). Hence, it would be interesting to investigate the role of PRRs in T cells during other parasitic infections to identify pathways that could possibly be exploited for therapeutic purposes.

CONCLUDING REMARKS

An important body of literature has now demonstrated that PRRs are expressed and functional in cells of the adaptive immune system. In these cells, PRR activation can support signaling pathways that are beneficial to host immunity or, on the contrary, promote adverse effects that favor pathogen persistence. In light of this information, it is thus important to consider the effect of TLR agonists on T and

B cells, and not only on myeloid cells, when developing new vaccination strategies, particularly for therapeutic purposes. Indeed, endosomal TLR agonists' administration during chronic stages of infection may lead to CD4 T cell death and/or exacerbate hypergammaglobulinemia. The same caveat may also be valid for immunotherapeutic interventions involving TLR agonists, which could have disease-exacerbating consequences if the wrong cells are involuntarily targeted.

Further investigations are required to better define signaling pathways and downstream targets of PRRs in T and B cells in the context of chronic infections, since these pathways could be exploited for novel therapies.

AUTHOR CONTRIBUTIONS

TS and SS made substantial, direct, and intellectual contribution to the work and wrote the manuscript.

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Influence of the Draining Lymph Nodes and Organized Lymphoid Tissue Microarchitecture on Susceptibility to Intradermal *Trypanosoma brucei* Infection

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Infection of the mammalian host with African trypanosomes begins when the tsetse fly vector injects the parasites into the skin dermis during blood feeding. After injection into the skin, trypanosomes first accumulate in the draining lymph node before disseminating systemically. Whether this early accumulation within the draining lymph node is important for the trypanosomes to establish infection was not known. Lymphotoxin-β-deficient mice ($LT\beta^{-/-}$ mice) lack most secondary lymphoid tissues, but retain the spleen and mesenteric lymph nodes. These mice were used to test the hypothesis that the establishment of infection after intradermal (ID) *T. brucei* infection would be impeded in the absence of the skin draining lymph nodes. However, $LT\beta^{-/-}$ mice revealed greater susceptibility to ID *T. brucei* infection than wild-type mice, indicating that the early accumulation of the trypanosomes in the draining lymph nodes was not essential to establish systemic infection. Although $LT\beta^{-/-}$ mice were able to control the first parasitemia wave as effectively as wild-type mice, they were unable to control subsequent parasitemia waves. $LT\beta^{-/-}$ mice also lack organized B cell follicles and germinal centers within their remaining secondary lymphoid tissues. As a consequence, $LT\beta^{-/-}$ mice have impaired immunoglobulin (Ig) isotype class-switching responses. When the disturbed microarchitecture of the B cell follicles in the spleens of $LT\beta^{-/-}$ mice was restored by reconstitution with wild-type bone marrow, their susceptibility to ID *T. brucei* infection was similar to that of wild-type control mice. This effect coincided with the ability to produce significant serum levels of Ig isotype class-switched parasite-specific antibodies. Thus, our data suggest that organized splenic microarchitecture and the production of parasite-specific Ig isotype class-switched antibodies are essential for the control of ID African trypanosome infections.

Keywords: ***Trypanosoma brucei*, trypanosomes, spleen, lymph nodes, B cells, lymphotoxin**

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INTRODUCTION

African trypanosomes are single-celled protozoan parasites that are transmitted between mammalian host species via blood-feeding tsetse flies of the genus *Glossina*. The *Trypanosoma brucei rhodesiense* and *T. b. gambiense* subspecies cause human African trypanosomiasis in endemic regions of sub-Saharan Africa. Animal African trypanosomiasis is caused by

Trypanosoma congolense, *Trypanosoma vivax* and *T. brucei*, and inflicts substantial economic strains on the African livestock industry. These parasites exist entirely extracellularly within their hosts, and the life cycle in the mammalian host is initiated by the intradermal (ID) injection of metacyclic trypomastigotes into the skin dermis by the tsetse fly vector. After ID injection the parasites can directly infect the lymphatics (1) before disseminating to the draining lymph nodes and then systemically via the bloodstream (2–5). Soon after ID injection the parasites also undergo morphological change into the long-slender bloodstream form stages that are specifically adapted for survival within the hostile environment of the mammalian bloodstream.

Although much attention has been given to the study of experimental trypanosome infections initiated by intraperitoneal (IP) or intravenous injection, comparatively little is known of the host-specific factors that are important for the effective control of ID transmitted trypanosome infections. However, Wei and colleagues have shown that infection of mice by the IP route establishes a detectable parasitemia earlier than after ID injection, with a higher parasite burden (6). Furthermore, the same study suggested mice were ∼100X less susceptible to ID injected *T. brucei* infection when compared to mice infected by the IP route.

Lymphotoxin-α (LTα) and lymphotoxin-β (LTβ) are members of the TNF superfamily and form a heterotrimer ($LT\alpha_1\beta_2$) to allow signaling through the LTβ receptor (LTβR) (7). Signaling via LTβR during embryonic development is essential for the development of the peripheral lymph nodes (8). Subsequently, post-natal lymphocyte-derived $LT\alpha_1\beta_2$-mediated stimulation is also essential for the maintenance of organized tissue microarchitecture within the secondary lymphoid organs. As a consequence, mice deficient in LTα, LTβ, or LTβR lack most peripheral lymph nodes and have grossly disorganized microarchitecture in their remaining secondary lymphoid organs such as the spleen (8–15). For example, these mice have disturbed B cell follicles, lack germinal centers (GC) and as a consequence have a significantly reduced ability to produce high affinity antigen-specific class-switched immunoglobulins (Ig) upon immunization. Therefore, in this study we used $LT\beta^{-/-}$ mice that lack most peripheral lymph nodes to determine the requirement of the draining lymph nodes for the effective establishment of infection after intradermal (ID) inject with *T. brucei*. Using these mice we show that the early accumulation of the trypanosomes in the draining lymph nodes was not essential to establish systemic infection. Although $LT\beta^{-/-}$ mice were able to control the first parasitemia wave as effectively as wild-type mice, they were unable to control subsequent parasitemia waves. Subsequent experiments suggested that the inability of $LT\beta^{-/-}$ mice to control the subsequent parasitemia waves coincided with the lack of organized B cell follicles in their spleens, and their impaired ability to mount parasite-specific Ig isotype class-switched antibody responses. This study provides important insight into the important host factors that are essential for the efficient control of ID trypanosome infections.

MATERIALS AND METHODS

Mice

Six to 8 weeks old female C57BL/6J mice (Charles Rivers, Harlow, England) and lymphotoxin-β-deficient ($LT\beta^{-/-}$) mice (15) were used throughout this study. Mice were maintained in individually ventilated cages and provided food and water *ad libitum*. All procedures using experimental mice were carried out under the authority of the appropriate project and personal licenses, in accordance with the United Kingdom Home Office regulations and the Animals (Scientific Procedures) Act 1986. Ethical approvals were obtained from The Roslin Institute's and University of Edinburgh's ethics committees.

γ-Irradiation and Reconstitution With Donor Bone Marrow

Where indicated, recipient female $LT\beta^{-/-}$ mice (∼20 g each, 6–8 weeks old) were γ-irradiated twice (5 Grays each) at a 4 h interval. Bone marrow from the long bones of donor mice was aseptically prepared as a single cell suspension at $\sim 1 \times 10^7$ cells/ml in HBSS (Life Technologies, Paisley, UK). Approximately 18 h after the irradiation the recipient mice each received 100 μl of fresh donor bone marrow by intravenous injection into the tail vein. Recipient mice were allowed to recover for 10 weeks prior to their use in subsequent experiments.

Trypanosomes

The pleomorphic wild-type *T. b. brucei* strain STIB247 was used throughout this study. These trypanosomes were originally isolated from a hartebeeste (*Alcelaphus buselaphus*) in Tanzania's Serengeti National Park in 1971 (16). The trypanosomes were axenically cultivated *in vitro* as described previously (1). Prior to their use in the *in vivo* studies described, $\sim 1x10^5$ axenically cultivated trypanosomes were first injected IP into groups of C57BL/6J mice. Blood was collected at the peak of the first parasitemia and used as a fresh source of *in vivo*-adapted parasites for each experiment. In the experiments described the mice were infected ID with $\sim 1 \times 10^2$ or 1×10^5 *T. b. brucei* STIB247 parasites, where indicated.

Comparison of Serum Immunoglobulin (Ig) Isotype Levels by ELISA

Serum IgM and IgG isotype levels were measured by ELISA. For total Ig measurements a capture ELISA was used whereby 96 well plates (Immulon 4HBX 96-Well Micro Plate, SLS, UK) were first coated with 50 μl of either purified rat anti-mouse IgM coating antibody (BD 553435 (II/41), BD Biosciences, USA) or purified polyclonal goat anti-mouse coating Ig antibody (BD 553998, BD Biosciences, USA), each prepared at 5 μg/ml in p-nitrophenyl phosphate substrate buffer. The plates were sealed and incubated overnight at 4°C. The plates were then blocked using 100 μl of 1% bovine serum albumin (BSA)/PBS (Sigma-Aldrich) to each well and incubated at 37°C for 1 h, and then washed 5 times in 0.05% Tween/PBS. Sera (50 μl/well) was then added in 0.1% BSA/PBS and incubated for 1 h at 37°C at the following dilutions: IgM, 1:400; IgG1 1:800; IgG2c, 1:50; IgG3, 1:800. The serum

dilutions used were based on previously established titrations. Serial dilutions of normal mouse IgM (clone RMM-1), normal mouse IgG1 (clone RMG1-1), normal mouse IgG2c (clone RMG2a62) or normal mouse IgG3 (clone R40-82) (all from Biolegend, USA) were used to establish standard curves. After washing, 50 µl of Ig-subclass-specific biotinylated secondary antibodies (anti-IgM, clone G155-228, BD Biosciences; anti-IgG1, clone MG1-45, BioLegend; anti-IgG2c, clone MG2a-53, BioLegend; anti-IgG3, clone MG3-25, clone MG3-25) were applied in 1% skimmed milk/0.1% BSA/PBS (1/500 dilution), and incubated for 1 h at 37°C. Plates were subsequently washed and 50 µl streptavidin-conjugated horseradish peroxidase was added (1/1,000 dilution) and incubated at 37°C for 1 h. After a final wash, bound peroxidase activity was revealed by adding 50 µl of SureBlue TMB microwell Peroxidase Substrate to each well (KPL, SeraCare 5120-0075, Massachusetts, USA), and the reaction stopped using HCl (1M). Optical density (OD) was then determined using a Perkin Elmer Wallac 1420 Victor^2 Microplate Reader (GMI, USA) at 450 nm with 620 nm used as the reference OD value.

To estimate trypanosome-specific Ig levels an indirect ELISA was used whereby the plates were first coated with 50 µl of trypanosome lysate (0.7 µg protein/ml) in 0.1 M bicarbonate buffer, in place of the purified rat anti-mouse IgM purified polyclonal goat anti-mouse coating Ig antibodies. The remainder of the assay was repeated as above. A potential limitation of this approach being that the binding characteristics of the coating Ig antibodies used in the capture ELISA may differ from that of the trypanosome lysate used in the indirect assay.

Immunohistochemistry

Spleens were snap frozen at the temperature of liquid nitrogen, and 10 µm thick sections cut using a cryostat. Sections were then fixed in acetone and follicular dendritic cells detected using mAb 8C12 to detect CD35 (BD Biosciences, Oxford, UK) and B cells detected using rat anti-mouse B220 mAb (clone RA3-6B2, Life Technologies). Sections were subsequently stained with species-specific secondary antibodies conjugated to Alexa Fluor 488 (green) or Alexa Fluor 594 (red) dyes (Life Technologies). Sections were then imaged using a Zeiss LSM710 confocal microscope (Zeiss, Welwyn Garden City, UK).

Statistics

ELISA data were tested for normal distribution using Shapiro-Wilk and Kolmogorov Smirnov tests. Data that passed these normality tests were subsequently compared using a Student's *t*-test. Data that did not pass these tests were compared using a Mann-Whitney *U* test. These analyses were performed using GraphPad Prism v.8.0 (GraphPad Software Inc. San Diego, USA). To compare the parasitemias between different groups, linear mixed effects models were performed using RStudio. These were used to statistically compare the quadratic (squared) and cubic curve effect of the infected mouse parasitemia across the observation period. Mean peak parasitemias were compared using a Student's *t*-test or Tukey's multiple comparisons test. $P < 0.05$ were accepted as significant.

RESULTS

LTβ$^{-/-}$ Mice Lack Most Secondary Lymphoid Tissues

First, LTβ$^{-/-}$ mice and C57BL/6J wild-type (WT) control mice were injected IP with Chicago Sky Blue 6B ink and the macroscopic presence of their secondary lymphoid tissues determined at post-mortem 7 d later. Throughout this study the ear pinna was used as the site of ID parasite injection. The mandibular lymph nodes, sub-mandibular lymph nodes and superficial parotid lymph nodes are considered to drain this anatomical region. As anticipated, these lymph nodes were absent in LTβ$^{-/-}$ mice (**Table 1**) as stimulation through the LTβR during embryonic development is essential for their formation (12, 17, 18). However, the LTβ$^{-/-}$ mice retained the spleen (**Table 1**).

Increased Susceptibility of LTβ$^{-/-}$ Mice to Intradermal *T. brucei* Infection

Independent studies using the *T. brucei* strain 10–26 have estimated that the ID route of infection is ∼100X less efficient than the IP route (6). Although infected tsetse flies are likely to transmit low numbers of trypanosomes during blood feeding, ID injection of WT mice with a 1×10^2 dose of *T. brucei* strain 10–26 parasites was insufficient to establish infection (6). Here, WT control mice ($n = 8$/group) were also injected ID into the ear pinna with 1×10^2 *T. brucei* STIB 247 parasites, and blood

TABLE 1 | Incidence of lymphoid tissues in WT and LTβ$^{-/-}$ mice.

Lymphoid tissue	Mouse strain							
	WT				LTβ$^{-/-}$			
	M1	M2	M3	M4	M1	M2	M3	M4
Mandibular lymph node	2	2	2	2	0	0	0	0
Sub-mandibular lymph node	2	2	2	2	0	0	0	0
Superficial parotid lymph node	2	2	2	2	1	0	0	0
Spleen	1	1	1	1	0	0	0	0

Number of secondary lymphoid tissues detected macroscopically in wild-type (WT) and LTβ$^{-/-}$ mice. Each column represents an individual mouse (M), 4 mice/group.

parasitemias assessed daily for 30 d using the rapid matching method (19). As this assay has a detection threshold of $\sim 4 \times 10^5$ parasites/ml blood, parasitemias below this level were classified as below the limit of detection. Consistent with the above study, our data show that when WT mice were injected ID with 1×10^2 trypanosomes, only two of eight mice had a detectable parasitemia for short duration during the 30 d observation period (**Figure 1A**). Therefore, since mice are considered to be at least 100X less susceptible to ID *T. brucei* infection, in the subsequent experiments below the mice were injected ID with 1×10^5 trypanosomes to ensure that all the recipient WT mice developed a detectable parasitemia.

Next, groups of LTβ$^{-/-}$ mice and WT control mice ($n = 8$/group) were injected ID with 1×10^5 *T. brucei* STIB 247 parasites, and blood parasitemias assessed daily for 30 d. After infection with this dose of parasites all the mice developed a detectable parasitemia. Furthermore, the onset and duration of the first detectable parasitemia wave in the bloodstream of each mouse group was similar (**Figures 1B,C**): WT mice, 6–8 d post-infection (dpi); LTβ$^{-/-}$ mice, 5–7 dpi. Furthermore, the mean parasite burdens at the peak of the first parasitemia wave were also similar in each mouse group (**Figures 1B,C**): WT mice, 8×10^6/ml parasites/ml; LTβ$^{-/-}$ mice, 1×10^7 parasites/ml; $P = 0.460$, Student's *t*-test, $n = 8$/group.

None of the ID-injected WT mice displayed any relapse in their parasitemias during the remainder of the 30 d observation period. In contrast, five of eight of the LTβ$^{-/-}$ mice relapsed between 21 and 30 dpi, displaying subsequent parasitemia waves (**Figures 1B,C**). These data clearly show that ID injected *T. brucei* can successfully establish infection in the bloodstream of LTβ$^{-/-}$ mice despite the absence of the draining lymph nodes. However, the LTβ$^{-/-}$ mice have a reduced ability to control an ID *T. brucei* infection when compared to WT mice.

LTβ$^{-/-}$ Mice Have Disturbed Splenic Microarchitecture and Reduced Serum Class-Switched Immunoglobulin Levels Following Trypanosome Infection

Constitutive post-natal LTβR-stimulation is also important for the maintenance of organized B cell follicles and the stromal follicular dendritic cells (FDC) within them (20). As a consequence, the spleens of LTβ$^{-/-}$ mice lacked CD35-expressing FDC and had disturbed B cell follicles that presented as ring-like structures (**Figure 2A**).

The retention of antigens on the surface of FDC is essential for GC formation and the production of high-affinity antigen-specific immunoglobulin (Ig) isotype class-switched antibodies by B cells (21). The levels of total IgM, IgG1, IgG2c, and IgG3 antibodies in the serum of uninfected naïve WT and LTβ$^{-/-}$ mice were similar (**Figure 2B**), consistent with previous data (10). By 30 d following ID infection with *T. brucei*, the sera of infected mice from each group contained significantly elevated levels of trypanosome-specific IgM (**Figure 2C**). However, whereas elevated levels of trypanosome-specific class-switched IgG1, IgG2c and IgG3 antibodies were detected in the sera of WT mice, significantly lower levels, if any, were detected in the sera of LTβ$^{-/-}$ mice (**Figures 2D–F**).

WT Bone Marrow Restores Splenic Microarchitecture in LTβ$^{-/-}$ Mice and Reduces Their Susceptibility to ID *T. brucei* Infection

We next determined whether the increased susceptibility of LTβ$^{-/-}$ mice to ID *T. brucei* infection was a consequence of their impaired ability to produce trypanosome-specific class-switched Ig isotypes. Constitutive LTβR-mediated stimulation via lymphocyte-derived $LT\alpha_1\beta_2$ is essential for the maintenance of FDC networks and lymphoid tissue microarchitecture (18, 20, 22). As mature FDC are important for the efficient production of antigen-specific class-switched antibodies by B cells (21, 23), FDC differentiation was induced in the remaining lymphoid tissues of LTβ$^{-/-}$ mice. Thus, the reconstitution of LTβ$^{-/-}$ mice with LT-expressing donor bone marrow from WT mice (termed WT→ LTβ$^{-/-}$ mice, hereafter) stimulated the differentiation of FDC from stromal-derived precursor cells in the spleens of the recipient LTβ$^{-/-}$ mice (**Figure 3**). However, this treatment does not induce the development of the missing secondary lymphoid tissues (24). Conversely, when LTβ$^{-/-}$ mice were reconstituted with LTβ$^{-/-}$ donor bone marrow as a negative control (termed LTβ$^{-/-}$→ LTβ$^{-/-}$ mice, here after), no induction of FDC network formation was observed (**Figure 3**).

Next, groups of WT→ LTβ$^{-/-}$ mice, LTβ$^{-/-}$→ LTβ$^{-/-}$ mice and un-irradiated WT control mice were ID injected with 1×10^5 *T. brucei* STIB 247 parasites and blood parasitemias assessed daily for 30 d. The onset and duration of the first detectable parasitemia waves in the bloodstream of each mouse group were similar (**Figures 4A,B**). However, noticeable differences in the parasitemia kinetics were evident during the later stages of the infections. The trypanosome infection relapsed in most of the LTβ$^{-/-}$→ LTβ$^{-/-}$ mice from ∼21 dpi with the detection of subsequent parasitemia waves (**Figures 4A,B**). These data show that LTβ$^{-/-}$→ LTβ$^{-/-}$ mice, like LTβ$^{-/-}$ mice (**Figure 1**), have increased susceptibility to ID *T. brucei* infection. In contrast, none of the WT→ LTβ$^{-/-}$ mice, and only one of eight of the ID injected un-irradiated WT mice, had any detectable relapses in their parasitemias during the remainder of the experiment (**Figures 4A,B**).

Reconstitution of LTβ$^{-/-}$ Mice With WT Bone Marrow Induces Their Ability to Produce Ig Isotype Class-Switched Antibodies

We next determined whether the reconstitution of LTβ$^{-/-}$ mice with WT bone marrow induced the ability to produce class-switched Ig isotypes. By 30 d following ID injection with *T. brucei*, similarly elevated levels of total IgM were detected in the sera of WT mice, WT→ LTβ$^{-/-}$ mice and LTβ$^{-/-}$→ LTβ$^{-/-}$ mice (**Figure 5A**). However, the sera of WT→ LTβ$^{-/-}$ mice contained significantly elevated levels of total class-switched IgG isotypes when compared to uninfected WT controls (**Figures 5B–D**). These data suggest that the restoration of splenic microarchitecture in WT→ LTβ$^{-/-}$ mice coincided with the ability to produce class-switched Ig isotypes.

Finally, we compared the levels of trypanosome-specific antibodies in the sera of infected WT mice, WT→ LTβ$^{-/-}$ mice and LTβ$^{-/-}$ → LTβ$^{-/-}$ mice. Sera from infected mice from each

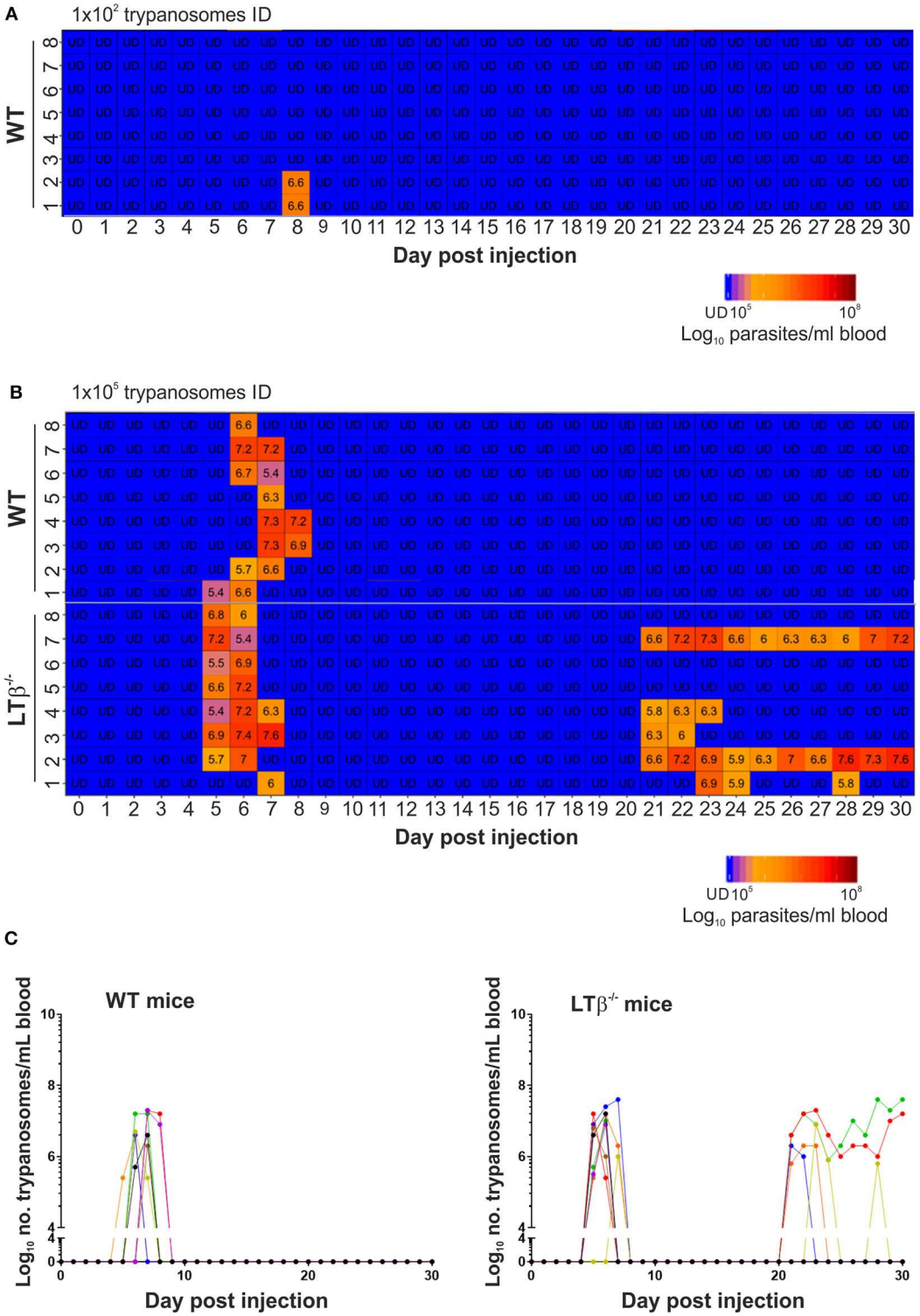

FIGURE 1 | Enhanced susceptibility of LTβ$^{-/-}$ mice to ID infection with *T. brucei*. **(A)** Wild-type (WT) mice ($n = 8$) were injected ID with a 1×10^2 dose of *T. brucei* STIB 247 parasites and blood parasitemia levels determined at daily intervals. Heatmap shows the blood parasitemia ($\log_{10}$ number of trypanosomes/ml of blood) in each mouse. Each row represents data from an individual mouse. UD = below the limit of detection, ~5.4 $\log_{10}$ parasites/ml. **(B)** Groups of WT and LTβ$^{-/-}$ mice ($n =$ 8 mice/group) were injected ID with a 1×10^5 dose of *T. brucei* STIB 247 parasites and blood parasitemia levels determined at daily intervals. Heatmap shows the blood parasitemia ($\log_{10}$ number of trypanosomes/ml of blood) in each mouse. Each row represents data from an individual mouse. UD, below the limit of detection, ~5.4 $\log_{10}$ parasites/ml. **(C)** Charts show parasitemia profiles ($\log_{10}$ number of trypanosomes/ml of blood) in each mouse from each group following ID injection with 1×10^5 dose of T. brucei STIB 247 parasites. Each line represents data from an individual mouse.

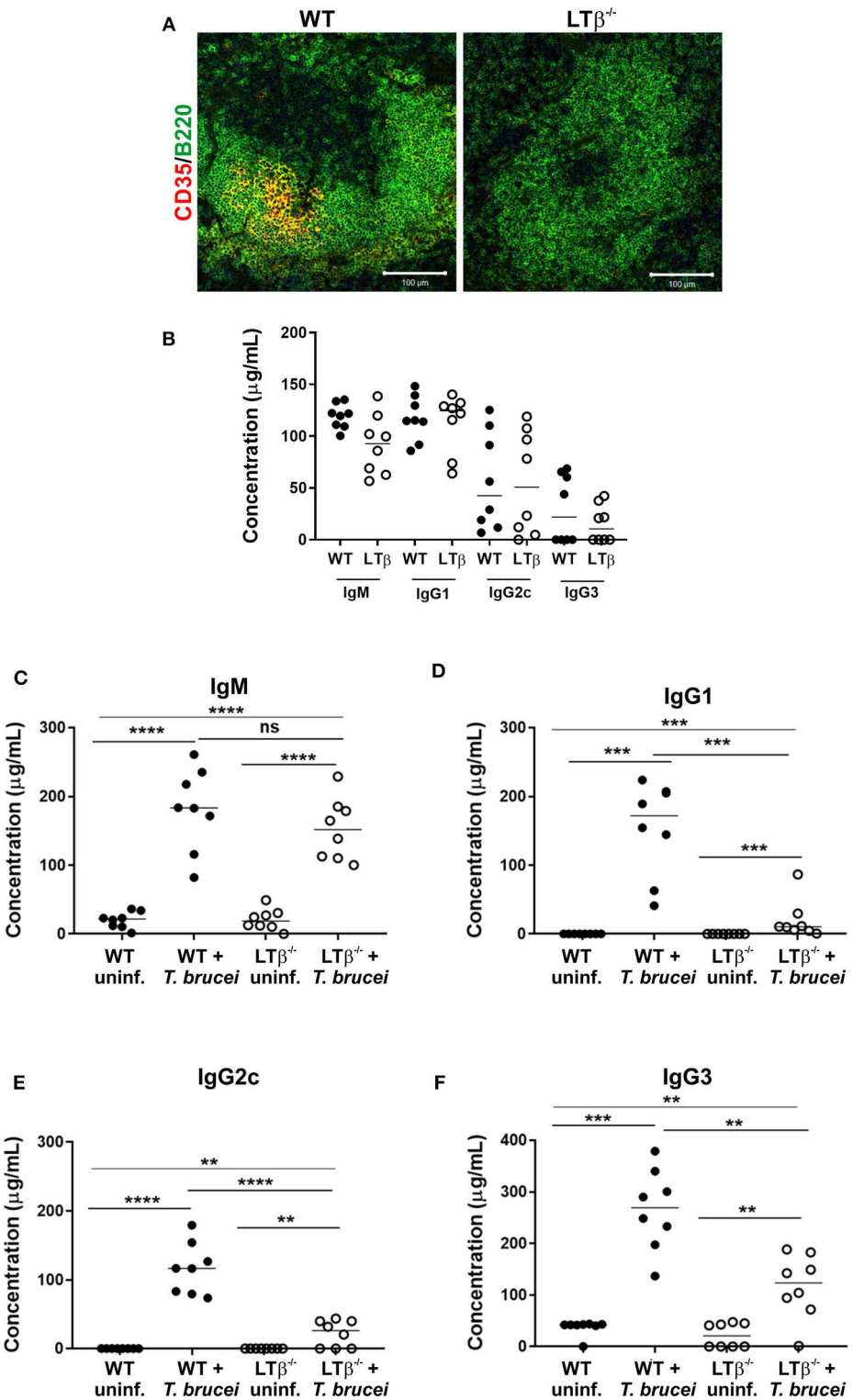

FIGURE 2 | LTβ$^{-/-}$ mice have disturbed splenic microarchitecture and reduced serum class-switched immunoglobulin levels after trypanosome infection. **(A)** Spleen sections from wild-type (WT) and LTβ$^{-/-}$ mice were immunostained to detect follicular dendritic cells (FDC, CD35+ cells, red) and B cells (B220+ cells, green). The spleens of LTβ$^{-/-}$ mice lacked FDC networks and had disturbed B cell follicles. **(B)** Total serum IgM, IgG1, IgG2c, and IgG3 concentrations in uninfected naïve WT and LTβ$^{-/-}$ mice determined by ELISA ($n = 8$ mice/group). **(C–F)** Groups of WT and LTβ$^{-/-}$ mice ($n = 8$ mice/group) were injected ID with a 1×10^5 dose of *T. brucei* STIB 247 parasites and serum concentrations of trypanosome-specific **(C)** IgM, **(D)** IgG1, **(E)** IgG2c and **(F)** IgG3 determined by ELISA. Uninf., uninfected. Closed circles, WT mice; open circles, LTβ$^{-/-}$ mice. Each point represents data from an individual mouse. Horizontal bar, median. Statistical analyses: **(B)** Student's *t*-test; **(C)** Student's *t*-test; **(D)** Mann-Whitney *U*-test; **(E)** Student's *t*-test; **(F)** Mann-Whitney *U*-test; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

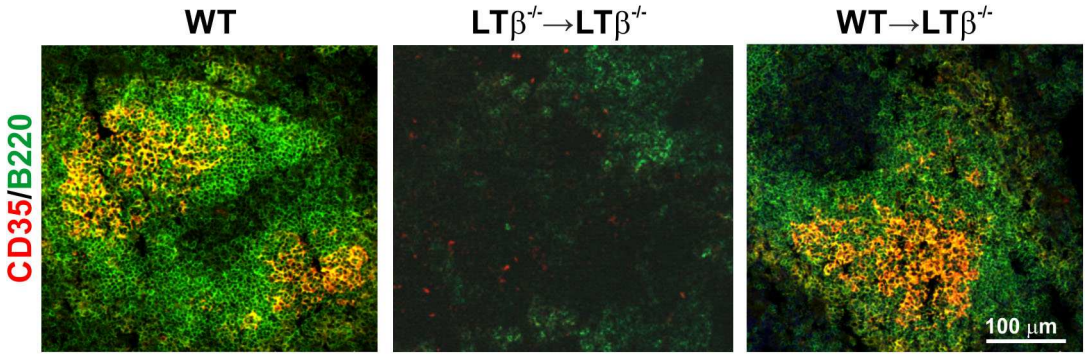

FIGURE 3 | Reconstitution of LTβ$^{-/-}$ mice with wild-type (WT) bone marrow restores the disturbed splenic microarchitecture. LTβ$^{-/-}$ mice were γ-irradiated and reconstituted with donor bone marrow from WT mice (WT→ LTβ$^{-/-}$ mice) or LTβ$^{-/-}$ mice (LTβ$^{-/-}$→ LTβ$^{-/-}$ mice). Ten weeks later spleens were collected and sections immunostained to detect follicular dendritic cells (FDC, CD35+ cells, red) and B cells (B220+ cells, green). FDC differentiation was restored in the spleens of WT→ LTβ$^{-/-}$ mice.

group contained significantly elevated levels of trypanosome-specific IgM (**Figure 6A**). However, whereas WT mice and WT→ LTβ$^{-/-}$ mice produced similarly elevated levels of trypanosome-specific class-switched IgG1, IgG2c, and IgG3 antibodies, much lower levels if any were detected in the sera of LTβ$^{-/-}$ → LTβ$^{-/-}$ mice (**Figures 6B,C**). Thus, these data suggest that the restoration of the microarchitecture in the spleens of WT→ LTβ$^{-/-}$ mice enabled the production of trypanosome-specific class-switched antibodies.

DISCUSSION

Following ID injection into the host's skin the extracellular trypanosomes invade the afferent lymphatics (1) from where they infect the draining lymph nodes and begin to disseminate systemically (4, 5). For example, during the initial period following ID infection of cattle and goats with *T. vivax* by tsetse fly bite, the parasites were first encountered in the draining pre-scapular lymph nodes before the bloodstream (2, 3). Similar disease kinetics have also been reported in mice infected with *T. brucei* by tsetse fly bite (5). Circulating lymphocytes/leukocytes, antigens and other components within the lymph fluid can enter the bloodstream via the right lymphatic duct on the right side of the body, or the thoracic duct on the left side of the body. From these lymphatic ducts, the passage of lymph into the blood circulatory system is achieved either via the right or left subclavian vein. We show that the ability of the ID injected trypanosomes to establish systemic infection was not compromised in mice lacking the draining lymph nodes. Our data therefore suggest that following their initial invasion into the host's lymphatics, the trypanosomes can directly enter the bloodstream via the lymphatic ducts and disseminate further without the requirement for prior adaptation or amplification within the draining lymph nodes.

Although the LTβ$^{-/-}$ mice lacked most peripheral lymph nodes, had disorganized microarchitecture within their spleens and lacked the ability to mount trypanosome-specific class-switched IgG responses, the magnitude and duration of the initial parasitemia wave was similar when compared to WT mice. The increased ability of C57BL/6 mice to control the first parasitemia wave when compared to C3H/He mice, has been suggested to be associated with their relative ability to produce trypanosome-specific Ig (25). During the first few days after trypanosome infection the initial and predominant parasite-specific antibodies that are produced by the host are of the IgM isotype that recognize parasite surface antigens (26–29). The production of parasite-specific IgM was shown to be important for control of *T. b. evansi* infection (30). In contrast, a study using IgM-deficient mice has suggested only a limited role for IgM in controlling infection with clonal pleomorphic *T. brucei* AnTat 1.1E parasites (31). However, the compensatory effects of other Ig isotypes in host protection could not be entirely excluded. Macrophages have also been suggested to play an important role in the innate immune system's ability to control the infection by phagocytosing and destroying the parasites in tissues (32). For example, the Kupffer cells in the liver can aid the elimination of *T. congolense* from the bloodstream through IgM- and IgG-dependent phagocytosis of the parasites (33). In the current study the levels of parasite-specific IgM detected in the sera of LTβ$^{-/-}$ mice were similar to those in WT controls. This implies that a combination of parasite-specific IgM and the responses and actions of innate phagocytes are likely to play an important role in controlling the initial phase of the infection to the infecting parasite clones via antibody-dependent phagocytosis. It remains to be determined whether B1 B-cell subsets or B2 B-cell subsets, including GC B-cell, follicular B-cell and marginal zone (MZ) B cells, are the sources of the parasite-specific IgM in the current study. However, since mice that lack LTβR-signaling also lack GC, B cell follicles and MZ in their spleens (34, 35), B2 B cells are unlikely to be a major source of trypanosome-specific IgM in the ID infected LTβ$^{-/-}$ mice. An independent study has also suggested that MZ B cells are depleted following infection with trypanosomes by the IP route (36). The requirement for GC B cells is less clear. Whereas,

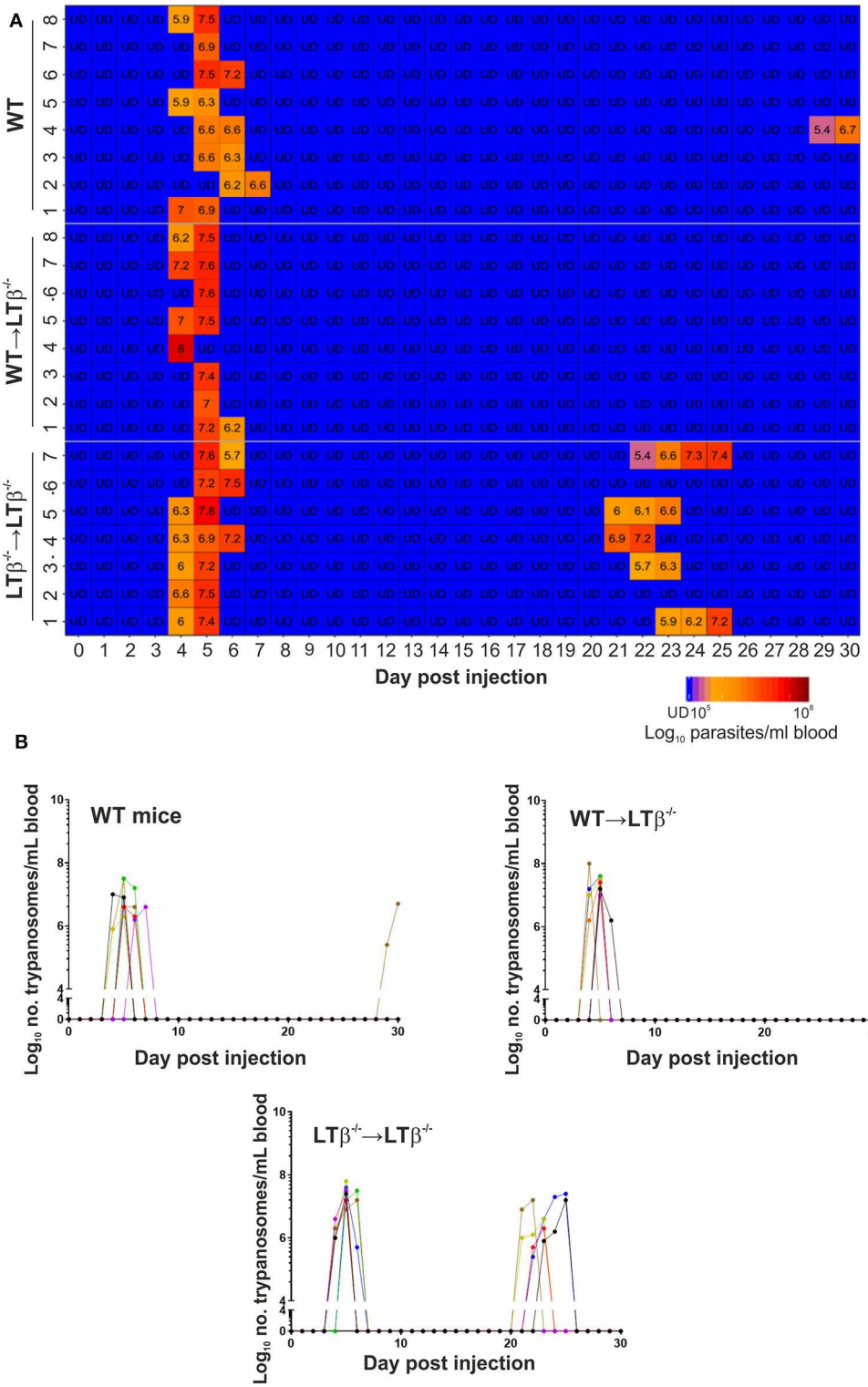

FIGURE 4 | Enhanced susceptibility of LTβ$^{-/-}$ mice to ID infection with *T. brucei*. Groups of LTβ$^{-/-}$ mice were γ-irradiated and reconstituted with donor bone marrow from wild-type (WT) mice (WT→ LTβ$^{-/-}$ mice) or LTβ$^{-/-}$ mice (LTβ$^{-/-}$ → LTβ$^{-/-}$ mice). Ten weeks later groups of WT mice, WT→ LTβ$^{-/-}$ mice and LTβ$^{-/-}$ → LTβ$^{-/-}$ mice (n = 7–8 mice/group) were injected ID with a 1 × 10^5 dose of *T. brucei* STIB 247 parasites and blood parasitemia levels determined at daily intervals. **(A)** Heatmap shows the blood parasitemia ($\log_{10}$ number of trypanosomes/ml of blood) in each mouse. Each row represents data from an individual mouse. UD, below the limit of detection, ~5.4 $\log_{10}$ parasites/ml. **(B)** Charts show parasitemia profiles ($\log_{10}$ number of trypanosomes/ml of blood) in each mouse from each group following ID injection with 1 × 10^5 dose of *T. brucei* STIB 247 parasites. Each line represents data from an individual mouse.

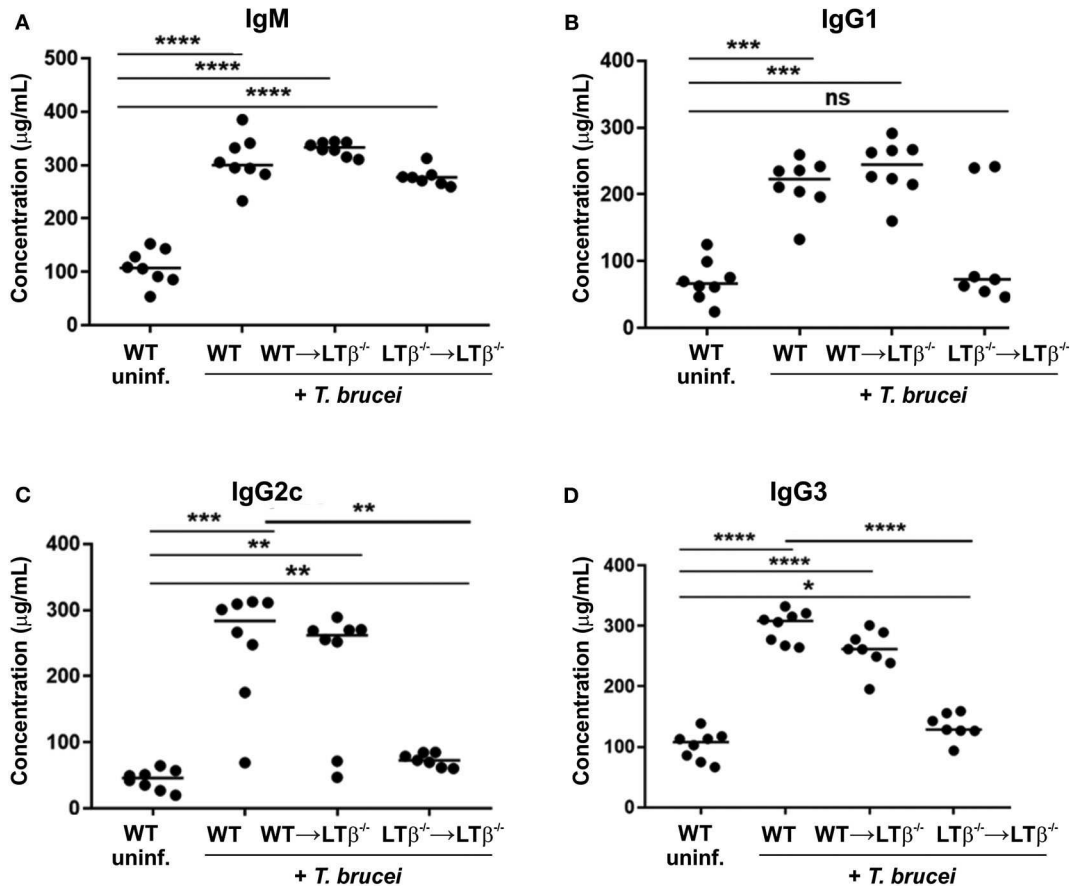

FIGURE 5 | Reconstitution of LTβ$^{-/-}$ mice with wild-type (WT) bone marrow restores their ability to produce Ig isotype class-switched antibodies. Groups of LTβ$^{-/-}$ mice were γ-irradiated and reconstituted with donor bone marrow from WT mice (WT→ LTβ$^{-/-}$ mice) or LTβ$^{-/-}$ mice (LTβ$^{-/-}$→ LTβ$^{-/-}$ mice). Ten weeks later groups of WT mice, WT→ LTβ$^{-/-}$ mice and LTβ$^{-/-}$ → LTβ$^{-/-}$ mice (n = 7–8 mice/group) were injected ID with 1x10^5 dose of *T. brucei* STIB 247 parasites and 30 d later concentrations of total serum **(A)** IgM, **(B)** IgG1, **(C)** IgG2c, and **(D)** IgG3 determined by ELISA. Uninf., uninfected. Each point represents data from an individual mouse. Horizontal bar, median. Statistical analyses: **(A)** Student's *t*-test; **(B)** Mann-Whitney *U*-test; **(C)** Mann-Whitney U test; **(D)** Student's *t*-test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

data suggest that GC are not induced in the spleen during trypanosome infections (37), GC B cells have been reported to accumulate in this tissue during the later stages of IP infection with pleomorphic *T. brucei* AnTat 1.1E parasites (36). In contrast, GC formation has been described in the spleens of mice after IP infection with *T. congolense* (38). Whether GC are also induced in the skin draining lymph nodes during *T. brucei* infection is not known.

Closer analysis suggested that the onset of the first parasitemia wave in the ID infected LTβ$^{-/-}$ mice typically occurred a day earlier than ID infected WT mice. The precise reason for this earlier onset is uncertain, but it is plausible that after ID infection in WT mice some of the parasites are initially retained in, or migration is slowed by, the local draining lymph node. Alternatively, some parasites may also be phagocytosed and destroyed by resident macrophages as they travel through the draining lymph node. Since LTβ$^{-/-}$ mice lacked the draining lymph nodes it is possible that these effects were avoided, enabling a higher burden of parasites to initially enter the blood-stream, leading to the slightly earlier development of the first parasitemia peak.

As the infection progressed the LTβ$^{-/-}$ mice were unable to successfully control the parasitemia when compared to WT mice. Following the initial parasitemia wave the production of trypanosome-specific Ig isotype class-switched IgG antibodies is essential for the control of the subsequent relapses in the parasitemia (29, 31, 39, 40). The parasite class-switched IgG antibodies that are induced during this phase of the disease target the trypanosome's variable surface glycoprotein coat with high affinity (40, 41). Furthermore, differences in the magnitude of the host's parasite-specific class-switched IgG response can have a direct influence on the pathogenesis of *T. congolense* infection in mice (42) and cattle (43). For example, relatively resistant C57BL/6 mice produce high levels of trypanosome-specific IgG isotypes when compared to relatively susceptible A/J mice (42).

Here, the increased susceptibility of LTβ$^{-/-}$ mice to ID *T. brucei* infection coincided with significantly reduced serum levels of parasite-specific class-switched Ig isotypes. Since stimulation

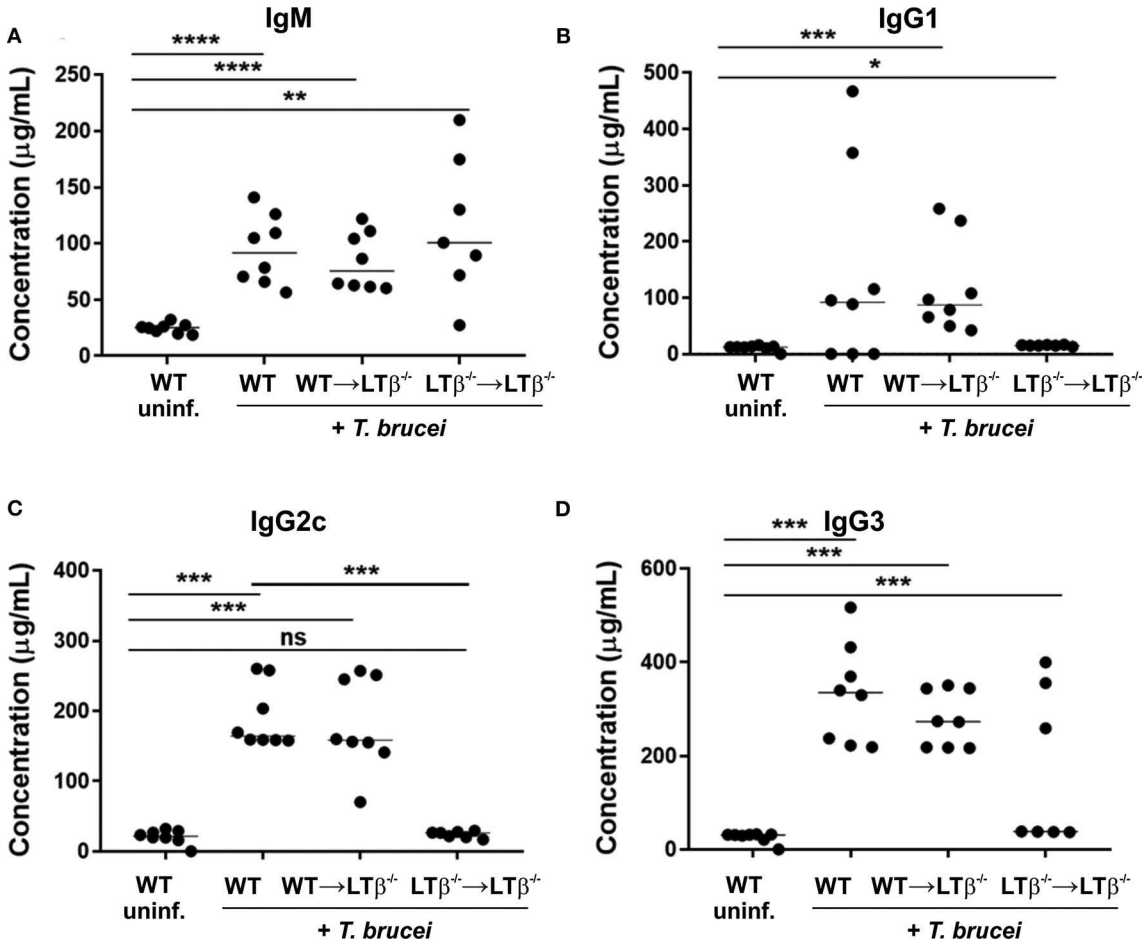

FIGURE 6 | Reconstitution of LTβ$^{-/-}$ mice with wild-type (WT) bone marrow restores their ability to produce trypanosome-specific Ig isotype class-switched antibodies. Groups of LTβ$^{-/-}$ mice were γ-irradiated and reconstituted with donor bone marrow from WT mice (WT→ LTβ$^{-/-}$ mice) or LTβ$^{-/-}$ mice (LTβ$^{-/-}$ → LTβ$^{-/-}$ mice). Ten weeks later groups of WT mice, WT→ LTβ$^{-/-}$ mice and LTβ$^{-/-}$ → LTβ$^{-/-}$ mice (n = 7–8 mice/group) were injected ID with a 1 × 10^5 dose of *T. brucei* STIB 247 parasites and 30 d later concentrations of total serum **(A)** IgM, **(B)** IgG1, **(C)** IgG2c, and **(D)** IgG3 determined by ELISA. Uninf., uninfected. Each point represents data from an individual mouse. Horizontal bar, median. Statistical analyses: **(A)** Student's *t*-test; **(B)** Mann-Whitney *U* test; **(C)** Mann-Whitney *U* test; **(D)** Student's *t*-test; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

through the LTβR is essential for the organization of the B cell follicles within the secondary lymphoid tissues, LTβ$^{-/-}$ mice have disorganized B cell follicles (9, 12) and a significantly reduced ability to elicit high affinity antigen-specific class-switched IgG responses (35). Consistent with these studies the ID infected LTβ$^{-/-}$ mice also had significantly reduced serum levels of trypanosome-specific IgG1, IgG2c, and IgG3. This implies that the reduced ability of the LTβ$^{-/-}$ mice to effectively control the parasitemia during the later stages of the infection was at least in part a consequence of their reduced ability to produce parasite-specific class-switched IgG antibodies. To test this hypothesis the splenic microarchitecture and the ability of LTβ$^{-/-}$ mice to produce antigen-specific Ig class-switched antibodies was restored by reconstitution with bone marrow from WT donor mice. Our data clearly show that the WT→ LTβ$^{-/-}$ mice produced significant levels of trypanosome-specific class-switched IgG1, IgG2c and IgG3, and this coincided with the ability of the WT→ LTβ$^{-/-}$ mice to control further relapses in the parasitemia to a similar extent as WT mice. Future studies using transgenic mice with specific deficiencies in IgG1 (44) or IgG3 (45) will help resolve the individual roles of parasite-specific class-switched antibody isotypes in the control of ID *T. brucei* infections.

Our studies using LTβ$^{-/-}$ mice suggest that the presence of the draining lymph nodes has little, if any, impact on susceptibility to ID *T. brucei* infection. However, our data indicate that the status of the microarchitecture of the spleen has an important role in controlling the trypanosome infection. In the current study the disorganized microarchitecture in the spleens of LTβ$^{-/-}$ mice and LTβ$^{-/-}$→ LTβ$^{-/-}$ mice coincided with increased susceptibility to ID *T. brucei* infections. Conversely, the organized splenic microarchitecture in WT mice and WT→ LTβ$^{-/-}$ mice coincided with their increased ability to control subsequent parasitemia waves. These data are

consistent with data in an independent study, which showed that in the absence of the spleen, parasite-specific IgG2a/c and IgG3 responses were impaired and this coincided with increased susceptibility to *T. congolense* infection (40). Our data are also consistent with those in a study using mice lacking the B cell adaptor molecule Bam32 (38). When compared to WT controls, these mice also had increased disease severity, which correlated with impaired splenic GC formation and a diminished ability to produce parasite-specific IgG after IP *T. congolense* infection. Others, however, have suggested that the induction of anti-trypanosome antibody responses can occur independently of the lymph nodes and spleen, during experimental IP infection with *T. brucei* AnTat 1.1E parasites (46). Furthermore, another study suggested that LTβ$^{-/-}$ mice had reduced susceptibility to IP infection with *T. congolense* (47). The reason for the discrepancy between these studies and our own is uncertain, but may be related to differences in route of infection, parasite burden and parasite strains. When LTα$^{-/-}$ mice (as used by Magez and colleagues, 2002, (46) are immunized with low doses of T-cell-dependent antigens their ability to produce high affinity antigen-specific IgG1 is reduced. Conversely, when these mice are immunized with high doses of antigens they produce similar levels of high affinity antigen-specific IgG1 to WT mice (13, 34). Studies in mice also show that the route of trypanosome injection can have a significant influence on disease susceptibility and pathogenesis, with ID infections with *T. brucei* or *T. congolense* being 100X less efficient than IP infections (6). Using the *T. brucei* strain 10-26, Wei and colleagues showed that a detectable parasitemia was established in all mice injected with as few as 10^2 parasites by the IP route. However, the same dose was insufficient to establish a detectable infection by the ID route. Indeed, a detectable parasitemia was only observed in all of the recipient mice when injected ID with 10^4 parasites. Wei and colleagues also showed that when the mice were infected by the IP route the trypanosomes established a detectable parasitemia earlier than after ID injection, with a higher parasite burden (6). In the studies by Magez et al. (46) and Okwor et al. (47) the mice were similarly injected with trypanosomes by the IP route. Thus, it is plausible that the IP infections led to a significantly higher and earlier antigen burden in the LTα$^{-/-}$ mice enabling high affinity parasite-specific IgG responses to be induced despite the absence of organized splenic microarchitecture (13, 34). To resolve these issues additional studies are now required to definitively address the contributions of the skin draining lymph nodes and the spleen for the induction and maintenance of parasite-specific antibody responses after ID trypanosome infection. This could include comparison of parasitemia kinetics, disease susceptibility and parasite-specific B cell responses in intact and splenectomised WT mice and LTβ$^{-/-}$ mice.

When considered in the context of similar studies, our data imply that the first wave of the parasitemia after ID infection with *T. brucei* is predominantly controlled by innate mechanisms, most likely including non-class switched IgM and the engulfment of the parasites by phagocytes. However, control of the subsequent parasitemia waves requires organized B cell follicles in the spleen and the production of trypanosome-specific class-switched IgG. Drug resistance, as well as adverse side-effects, continue to significantly impact on the ability to successfully treat and control trypanosome infections. A more thorough understanding of the host factors that are essential for the efficient control of ID trypanosome infections could identify novel therapeutics and aid the development of protective vaccines.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by The Roslin Institute's and University of Edinburgh's ethics committees.

AUTHOR CONTRIBUTIONS

LM and NM conceived the study and obtained funding. OA, LM, and NM designed the study. OA, EP, and BB performed the experiments and acquired these data. All authors interpreted these data and contributed to the final version of the manuscript.

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Interferon Alpha Favors Macrophage Infection by Visceral *Leishmania* Species Through Upregulation of Sialoadhesin Expression

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Type I interferons (IFNs) induced by an endogenous *Leishmania* RNA virus or exogenous viral infections have been shown to exacerbate infections with New World Cutaneous *Leishmania* parasites, however, the impact of type I IFNs in visceral *Leishmania* infections and implicated mechanisms remain to be unraveled. This study assessed the impact of type I IFN on macrophage infection with *L. infantum* and *L. donovani* and the implication of sialoadhesin (Siglec-1/CD169, Sn) as an IFN-inducible surface receptor. Stimulation of bone marrow-derived macrophages with type I IFN (IFN-α) significantly enhanced susceptibility to infection of reference laboratory strains and a set of recent clinical isolates. IFN-α particularly enhanced promastigote uptake. Enhanced macrophage susceptibility was linked to upregulated Sn surface expression as a major contributing factor to the infection exacerbating effect of IFN-α. Stimulation experiments in Sn-deficient macrophages, macrophage pretreatment with a monoclonal anti-Sn antibody or a novel bivalent anti-Sn nanobody and blocking of parasites with soluble Sn restored normal susceptibility levels. Infection of Sn-deficient mice with bioluminescent *L. infantum* promastigotes revealed a moderate, strain-dependent role for Sn during visceral infection under the used experimental conditions. These data indicate that IFN-responsive Sn expression can enhance the susceptibility of macrophages to infection with visceral *Leishmania* promastigotes and that targeting of Sn may have some protective effects in early infection.

Keywords: *Leishmania*, sialoadhesin, CD169, macrophages, IFN-α, type I IFN

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INTRODUCTION

Leishmaniasis is a family of related protozoal diseases occurring in the New- and Old World and is caused by *Leishmania* parasites responsible for clinical features ranging from cutaneous, mucocutaneous to visceral manifestations. Visceral leishmaniasis (VL), also known as kala-azar, is a lethal neglected tropical disease caused by *Leishmania donovani* and *L. infantum* and responsible for ∼0.2–0.4 million cases each year (1). It is a vector-borne disease transmitted by the bites of infected female phlebotomine sand flies (2). In the vertebrate host, entry and survival inside myeloid cells are essential factors to complete its life cycle (3) and to enable dissemination to internal organs such as the liver, spleen and bone marrow (4).

Recent reports on New World cutaneous *L. guyanensis* infections revealed a considerable impact of exogenous IFN-inducing viruses and an endogenous *Leishmania* dsRNA virus (LRV1) on primary infection and reactivation in mice (5). LRV1 presence in clinical isolates of *L. braziliensis* has been associated to increased risk of treatment failure (6). LRV-sequences were also detected in an Iranian *L. infantum* clinical isolate from a patient unresponsive to antimonial treatment (7). Viral co-infections and presence of *Leishmania* RNA virus are therefore increasingly perceived as risk factors for pathogenicity of human leishmaniasis (5, 7–9). The virus appears to use the *Leishmania* exosomal pathway to reach the extracellular environment (10). The exacerbating features of LRV1 in *L. guyanensis* were linked to the induction of type I interferons which primarily occurred through stimulation of the endosomal Toll-Like Receptor 3 (TLR3) pathway by viral dsRNA in mice (11, 12). Type I IFN is known to trigger the expression of various so-called interferon-stimulated genes (ISGs) (13), including some that are involved in viral recognition and entry. Sialoadhesin (Sn, CD169, Siglec-1) is an ISG-gene product (14–17) expressed on macrophages, belonging to the Siglec (sialic acid binding Ig-lectin) family (18). Human and mice Sn share 72% sequence homology (19, 20) and, unlike other Siglecs, seem to lack tyrosine-based signaling motifs which suggests a primary role in cell-cell interactions rather than in cell signaling (21). During HIV-infection, Sn expression levels have been correlated with type I IFN levels and inflammatory disease progression *in vivo* in macaques (16). Regarding HIV-1, Pino et al. showed that IFN-α activated macrophages have an enhanced ability to capture HIV-1 via Sn recognition. These macrophages could fuel novel $CD4^+$ T cell infections and contribute to HIV-1 dissemination (22). Recent reports described that Sn recognizes the sialic acid moieties onto the *Leishmania* surface (3, 23, 24) and is responsible for phagocytosis during a *Leishmania* infection (3). Other pathogens such as *Campylobacter jejuni* (25), group B *Streptococcus* (26) and *Trypanosoma cruzi* (27) were also shown to be phagocytosed by macrophages using the Sn-sialic acid interaction. Sn becomes highly upregulated under conditions of IFN-α stimulation, for example, during viral or bacterial infections *in vitro* and *in vivo* (14, 16, 17). As such, co-infections and/or IFN-α stimulation may have an impact on the course and pathogenicity of a *Leishmania* infection (5).

The present study evaluated the role of type I IFN in the infection outcome of different *L. infantum* and *L. donovani* strains. IFN-α stimulated macrophages showed higher infection levels compared to control-treated macrophages. Since Sn expression was described to be upregulated by type I IFN, we further unraveled the role of Sn during *in vitro* and *in vivo* *Leishmania* infections.

MATERIALS AND METHODS

Ethics

The use of laboratory rodents was carried out in accordance to all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 01/01/2013, and the declaration of Helsinki in its latest version) and was approved by the ethical committee of the University of Antwerp, Belgium (UA-ECD 2014-17, UA-ECD 2017-04, UA-ECD 2015-90).

TABLE 1 | Overview of *Leishmania* isolates used and their respective origin.

Strain	Code	Origin
L. infantum		
ITMAP263	MHOM/MA/67/ITMAP263	Reference lab strain, originally isolated from VL-patient in Morocco
LEM3323	MHOM/FR/96/LEM3323	French field isolate from HIV-patient
LEM5159	MHOM/FR/2006/LEM5159	French field isolate from HIV-patient
LLM2346	MHOM/ES/2016/LLM-2346	Spanish field isolate
L. donovani		
Ldl82	MHOM/ET/67/L82	Reference lab strain, originally isolated from VL-patient in Ethiopia
LLM1599	MHOM/ET/2007/LLM-1599	Ethiopian field isolate
LLM1600	MHOM/ET/2007/LLM-1600	Ethiopian field isolate
L. major		
JISH118	MHOM/SA/85/JISH118	Saudi Arabian field isolate

Animals

Female C57BL/6 and BALB/c mice (6–8 weeks old) were used for the collection of bone marrow cells and for the *in vivo* bioluminescent imaging experiments. Female golden hamsters (body weight 100-120 g) were used as donors for the collection of *Leishmania* amastigotes. Animals were purchased from Janvier (France) and kept in quarantine for at least 5 days before starting the experiment. Sn-deficient mice ($Sn^{-/-}$ C57BL/6) were reared at our facilities from breeding pairs provided by UGent (Prof. Dr. Dirk Elewaut, Molecular Immunology and Inflammation Unit, VIB-UGent). Food for laboratory rodents and drinking water were available *adlibitum*.

Parasite Species/Strains

The different *L. infantum*, *L. donovani,* and *L. major* strains used in this paper for the *in vitro* infections are listed in **Table 1**. Promastigotes were routinely cultured in T25 culture flasks containing 5 mL of HOMEM medium (Invitrogen, UK) supplemented with 10% heat inactivated fetal bovine serum (iFBS). *Ex vivo* amastigotes of *L. infantum* ITMAP263 were obtained from the spleen of heavily infected donor hamsters and purified using two centrifugation steps as described elsewhere (28). The strains for the *in vivo* infections were the bioluminescent *L. infantum* MHOM/FR/96/LEM3323^{PpyRE9} and MHOM/MA/67/ITMAP263^{PpyRE9}, generated by the stable introduction of the red-shifted firefly luciferase PpyRE9 using the pLEXSY-hyg2.1 vector (29).

Purification of a Sn-Specific Bivalent Nanobody and Conventional Antibody

Anti-mouse Sn monoclonal antibodies (mAb) SySy94 were produced and purified as described previously (17). Anti-mouse Sn bivalent nanobody (Biv4.40 Nb, courtesy UGent) was produced in the periplasm of transformed *Escherichia coli.* The

bacterial expression clone was expanded in LB Broth medium containing 100 μg/mL ampicillin, 2 mM $MgCl_2$ and 0.1% D-glucose at 37°C and 200 rpm in a New Brunswick incubator shaker. The induction of protein synthesis was carried out at OD_{600} 0.6–0.9 by addition of 1 mM IPTG (isopropyl β-d-1-thiogalactopyranoside) and continued incubation at 28°C and 200 rpm. Bacterial pellets were collected by centrifugation (20 min at 4,400 × *g*) at 16 h post-induction. Periplasmic protein extracts were obtained by osmotic shock. In brief, cells were resuspended in ice cold TES buffer (Tris-HCl 0.2 M, EDTA 0.5 mM, sucrose 0.5 M) and kept for 1 h at 4°C while shaking. Subsequently, TES/4 was added to the cells for 2 h at 4°C while shaking. $MgCl_2$ was added to a final concentration of 12 mM and the periplasmic extract was obtained by using the supernatant after two ultracentrifugation steps: first 30 min at 7,860 × *g* and second 15 min at 18,050 × *g*. The extract was ½ diluted in binding buffer (20 mM sodiumphosphate, 0.5 M NaCl and 20 mM imidazole in miliQ) and was loaded onto a Histrap HP column using an Akta Prime plus (GE Healthcare Life Sciences). His-tagged Biv4.40 Nb was eluted using 20 mM sodiumphosphate, 0.5 M NaCl and 0.5 M imidazole. Next, a size exclusion chromatography was performed on a Superdex GF75 10–300 mm column in Gibco LPS-free cell culture grade PBS (ThermoFisher Scientific). Chromatography was performed at 0.5 mL/min with elution of Biv4.40 Nb (30 kDa) after ± 22 min (**Supplementary Figure 1A**).

Quality Assessment of the Sn-Binding Capacity of the Bivalent Nanobody

The binding capacity of myc-tagged Biv4.40 to Sn was assessed by immunofluorescent staining. Biv4.40 was added to Sn-expressing CHO cells and non-transfected CHO cells that served as negative controls (30). Cells were seeded onto coverslips and incubated at 37°C for 24 h. Biv4.40 was added to the cells on ice for 60 min at 1 μg/mL. Cells were fixed with 4% paraformaldehyde for 20 min at ambient temperature. After fixation, the secondary mouse-anti-myc antibody (R950-25, ThermoFisher Scientific) was added at 1 μg/mL. After three wash steps with PBS, the chicken anti-mouse Alexa Fluor 488 (ThermoFisher Scientific, A21200) antibody was added to the cells. Nuclei were stained with DAPI (Sigma-Aldrich). Images were obtained using an Axio Observer inverted microscope (Zeiss) equipped with a Compact Light Source HXP 120C and with filter sets 49 and 10 for DAPI and Alexa Fluor 488 fluorophores, respectively. Images were processed using Image J software (**Supplementary Figures 1B,C**).

in vitro Infections in Bone Marrow-Derived Macrophages

Bone marrow cells from tibia and femur were collected from wildtype and $Sn^{-/-}$ C57BL/6 and BALB/c mice as described previously (31). Red blood cell lysis was performed with ACK buffer for 3 mim. Bone marrow cells were incubated in a petri-dish with 10 mL RPMI-1640 culture medium (Gibco®, Life Technologies) enriched with 1% non-essential amino acids, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% L-glutamine, 10% iFCS and 15% L929 cell line supernatant containing macrophage colony stimulating factor (M-CSF) for 6 days at 37°C and 5% CO_2 (17). On the fourth day of incubation, bone marrow-derived macrophages were either or not stimulated with 50 IU/mL (for assessing dose-dependency) or the standard dose of 5.0 × 10^2 IU/mL IFN-α (PBL Assay Science, 12100) in a 10 mL petri dish. After incubation, cells were detached with PBS containing 2% 1.0 M HEPES and 1% 0.5 EDTA solution. Bone marrow cells were seeded in 96-well plates at 30,000 cells/well in 100 μL. After 24 h of attachment, cells were infected with *L. infantum* metacyclic promastigotes (multiplicity of infection 5:1) or *ex vivo* amastigotes (multiplicity of infection 20:1) in 100 μL RPMI-1640. In some experiments, extracellular parasites were maximally removed after 24 h of infection, using an established protocol by rinsing the cells 2 × with PBS and incubating the cells with RPMI supplemented with 2% heat-inactivated horse serum, 1% penicillin/streptomycin and 1% L-glutamine (32). After various time points post-infection, infected macrophages were fixed with methanol and stained with Giemsa. For each condition, the intracellular parasite burden was quantified microscopically in at least 50 macrophages for determination of the infection index: $\frac{\#\ amastigotes\ counted}{total\ \#\ macrophages\ counted}$ (33).

Flow Cytometric Analysis of Sn Expression

Bone marrow cells from tibia and femur were collected and either or not subjected to IFN-α stimulation. Liver cells were collected following a 10-min transcardial perfusion with Krebs-Henseleit solution at a flow rate of 100 mL/h. The gallbladder was removed. Livers were mechanically disrupted in 5 mL DMEM medium (Thermo Fisher) containing liver dissociation enzymes (Miltenyi Biotec) and the gentleMACS™ Dissociator (Miltenyi Biotec). After a 30' enzymatic digestion at 37°C, the cell suspensions were passed through a 100 μm filter. Cells were counted in a KOVA chamber® with trypan blue and resuspended to a concentration of 2.0 × 10^7 cells/mL. 50 μL (1.0 × 10^6 cells) of the cell suspension was used for analysis. Cells were kept on ice for 15 minutes and incubated with an Fc-blocking antibody (2.4G2, courtesy Dr. Benoît Stijlemans, VUB, Brussels). Next, cells were stained for 20 minutes with anti-mouse CD169 (Sn)-APC (3D6.112, Biolegend®) and the KC panel was supplemented with CD45-APC-Cy7 (30-F11, eBioscienceTM), F4/80-PE-Cy7 (BM8, eBioscience™) and antibodies Tim4-PerCP (RMT4-54, eBioscience™). Viobility 405/520 fixable dye (Miltenyi Biotec) was used for exclusion of dead cells. Flow cytometry was performed on a BD FACSCalibur® apparatus (for bone marrow cells) or MACSQUANT 10® apparatus (for KC) and data were analyzed using the FlowJo® software. The Kupffer cell (KC) gating strategy is presented in **Supplementary Figure 2**.

Evaluation of the Impact of Sn and Sialic Acids During *in vitro* Infections

The impact of the Sn-sialic acid interaction was assessed using various complementary approaches. *In vitro* infections were conducted in parallel as described above in bone marrow-derived macrophages from Sn-deficient mice. Alternatively, bone marrow-derived macrophages were pre-incubated with 10 μg/mL SySy94 or Biv4.40 for 1 h prior to infection. To fully

exclude any role for LPS despite the precautions taken, cells were treated in one experiment with 25 μg/mL of polymyxin B (81334, Sigma-Aldrich) prior to addition of the pharmacological inhibitors. Blocking of sialic acids on the surface of *Leishmania* parasites was performed by adding 5 μg/mL soluble Sn (5610-SL, Bio-Techne, R&D Systems) to the parasites 1 h prior to infection.

in vivo Infections With VL Strains

Wildtype and Sn-deficient mice were infected in the tail vein with 1.0×10^8 metacyclic promastigotes MHOM/FR/96/LEM3323^{PpyRE9} or MHOM/MA/67/ITMAP^{PpyRE9} in 100 μL RPMI. In some experiments, mice were stimulated subcutaneously (34) with 1,000 IU/g IFN-α 3 days prior to infection. In another experiment, mice were injected intraperitoneally with 4 μg/g Poly(I:C) at 3 days prior to infection, on the day of infection and weekly after infection (35). Poly(I:C) (Sigma-Aldrich, P1530) was freshly dissolved at 800 μg/mL in PBS and was first heated to 50°C for 5 min followed by cooling on ice to maximize annealing.

At different time points post-infection, mice were subjected to bioluminescent imaging in an IVIS® Spectrum *in vivo* Imaging System (PerkinElmer). Briefly, D-luciferin substrate (Promega, Benelux) was injected intraperitoneally (0.15 mg/g BW), followed by anesthesia for 3 min in an induction chamber with 2.5% isoflurane (IsoFlo®, Zoetis). Upon induction, mice were imaged in the IVIS® chamber for 10 min. Images were analyzed using LivingImage v4.3.1 within regions of interest (ROI) corresponding with the liver (29).

Cytokine Response Analysis

Blood samples were collected via the tail vein using heparinized capillaries (75 μL per capillary). Two capillaries were collected per mice. The blood was centrifuged at 20,000 × *g* for 10 min. The supernatant was stored at −80°C until further analysis. A custom panel of cytokines (mouse, IFN-γ, IL-6, IL-10, TNF-α, and KC/GRO) Multispot Assay System kit from MSD® (Mesoscale diagnostics) was used for the multiplex ELISA analysis according to the manufacturer's instructions.

Statistical Analyses

Mann–Whitney U, Kruskal–Wallis and ANOVA statistical tests were performed in GraphPad Prism 7, considering $p < 0.05$ as statistically significant. Graphs were prepared in GraphPad Prism 7.

RESULTS

IFN-α Induces a Higher Susceptibility of Macrophages to Infection With Visceral *Leishmania* Species

We evaluated the infection of different *L. infantum* and *L. donovani* promastigote laboratory strains and clinical isolates in bone marrow-derived macrophages either or not subjected to stimulation with IFN-α. Although the focus of this study was on VL species, the cutaneous *L. major* JISH118 strain was also included. The infection indices were elevated when IFN-α was added to macrophages of BALB/c ($p = 0.0072$) and C57Bl/6 mice ($p = 0.0480$) (**Figure 1A**). Although some strains seem to benefit more from the IFN-α induced effects than others, recorded infection indices were consistently higher in stimulated macrophages of LEM3323 in both mice species (BALB/c $p = 0.0180$, C57BL/6 $p = 0.0454$), resulting in the selection of this strain for the majority of the subsequent *in vitro* and *in vivo* infection experiments. *In vitro* effects on numbers of intracellular amastigotes were notable within 48 h (**Figure 1B**, $p < 0.05$). Interestingly, the increase in infection index was mainly due to the cumulative entry of extracellular parasites, rather than an accelerated replication of amastigotes (**Figure 1B**). Using the *L. infantum* ITMAP263 strain from which both promastigotes and hamster spleen-derived amastigotes were available, the impact of also the life cycle stage could be assessed. In contrast to the promastigote infections ($p = 0.0047$), no effect of IFN-α was observed on infections initiated with ITMAP263 amastigotes under the stated experimental conditions (**Figure 1C**). The effect of IFN-α was dose-dependent, with the infection index increasing with higher IFN-α concentrations (**Figure 1D**).

Upregulation of Sn Expression by IFN-α Enhances *Leishmania* Infection in Macrophages

To investigate the impact of IFN-α on Sn expression, a flow cytometric analysis was performed to detect Sn expression (**Figure 2A**). Incubation of bone marrow-derived macrophages for 2 days with IFN-α resulted in enhanced Sn expression (MFI = 95.6 ± 75.8) compared to the non-stimulated cells (MFI = 50.0 ± 29.8) and IFN-α stimulated $Sn^{-/-}$ cells (MFI = 42.9 ± 21.2).

Since Sn was described to contribute to *Leishmania* entry and multiplication in macrophages (3), the effect of IFN-α stimulation on infection was evaluated in wildtype and $Sn^{-/-}$ macrophages. While marked differences were observed in wildtype macrophages, infection indices did not significantly increase at 4 and 48 h post-infection ($p > 0.9999$) in stimulated bone marrow-derived macrophages originating from $Sn^{-/-}$ mice (**Figure 2B**). Trends were already notable within 4 h of infection, indicating that IFN-α affects the early infection processes.

The contribution of Sn to the effect of IFN-α was assessed using two different pharmacological inhibitors, a monoclonal anti-Sn antibody (SySy94) and a bivalent nanobody (Biv4.40 Nb) that lacks an Fc antibody domain (**Figures 2C,D**). Our findings show that pre-treatment of macrophages with Sn-specific antibodies or nanobodies partially abrogates the IFN-α induced effects resulting in a lower infection index 48 h post-infection with the different VL strains (SySy94: $p = 0.0037$; Biv4.40: $p = 0.0053$). Some effects of IFN-α and the pharmacological inhibitors were also noted in $Sn^{-/-}$ mice. Although precautions were taken to purify antibodies and nanobodies in LPS-free conditions, an additional experiment was performed to exclude an impact of LPS by including polymyxin B in the cell system. The same impact of the pharmacological inhibitors on the infection indices was observed in the presence of polymyxin B. To further confirm the role of the Sn-sialic acid interaction, an excess of soluble Sn was added to the parasites prior to infection in order to saturate the surface sialic acids

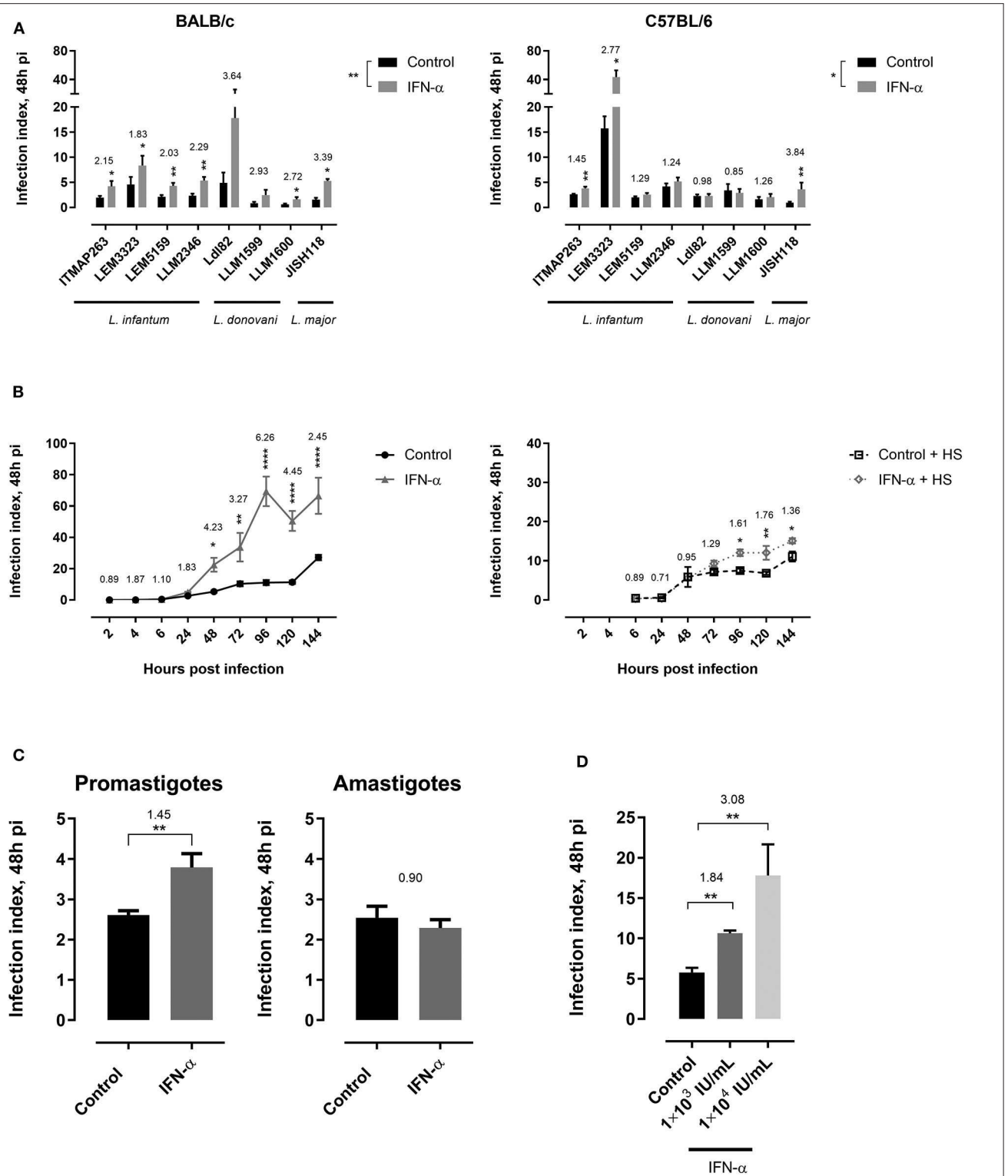

FIGURE 1 | Type I interferon induces a higher susceptibility of macrophages to infection with visceral *Leishmania* species. **(A)** Effect of IFN-α stimulation on the infection index of different *Leishmania* species/strains 48 h post-infection in bone marrow-derived macrophages from BALB/c (left) and C57BL/6 (right) mice. Cells were either left unstimulated (control) or were stimulated with IFN-α 2 days prior to infection with metacyclic promastigotes (5:1 infection ratio). Results in this panel are based on two experiments run in triplicate. **(B)** Effect of IFN-α stimulation on the infection index of *L. infantum* LEM3323 at different time points post-infection in

(Continued)

FIGURE 1 | bone marrow-derived macrophages from C57BL/6 mice. Cells were either left unstimulated (control) or were stimulated with IFN-α 2 days prior to infection with metacyclic promastigotes (5:1 infection ratio). Extracellular promastigotes were either washed (right panel) or not (left panel) with PBS 24 h after infection and cells were thereafter incubated with medium containing 2% horse serum (HS). Results in this panel are based on two experiments run in triplicate. **(C)** Infection index in bone marrow-derived cells of C57BL/6 mice either or not stimulated with IFN-α following infection with *L. infantum* ITMAP263 metacyclic promastigote or hamster spleen-derived amastigotes. Results in this panel are based on two experiments run in quadruplicate. **(D)** Effect of different doses of IFN-α stimulation on the infection index of LEM3323 48 h post-infection in bone-marrow derived macrophages from C57BL/6 mice. Results in this panel are based on two experiments run in triplicate. All results are expressed as mean ± standard error of mean (SEM) and the ratio of IFN-α/control is stated above the bars (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$).

(**Figure 2E**). A lower infection index ($p = 0.0013$) was obtained in the IFN-α stimulated condition when parasites were pretreated with the soluble Sn compared to the non-treated parasites.

Sn Plays a Moderate Role During *in vivo* VL Infections

Infection of *L. infantum* LEM3323^{PpyRE9} showed higher relative luminescent units in the liver of wildtype as compared to $Sn^{-/-}$ mice at 2 weeks post-infection ($p = 0.0454$) (**Figures 3A,B**) but was not associated with major changes in the serum cytokine levels (**Supplementary Figure 3**). Infection with *L. infantum* ITMAP263^{PpyRE9} in wildtype and $Sn^{-/-}$ mice did not show significant differences in liver burdens (**Supplementary Figure 4**), matching the *in vitro* observations of strain-dependent effects. Additional experiments were performed to mimic an antiviral response by combining the infection with a subcutaneous IFN-α stimulation (**Figure 3C**) or an exposure to Poly(I:C) (**Figure 3D**). No significant enhancement of infection was observed in response to these experimental triggers. The impact of these triggers on *in vivo* Sn expression was monitored on KCs (**Figure 3E**). KCs were found to already express steady-state levels of Sn which are unaffected by the IFN-α stimulation protocol. Poly(I:C) triggers enhanced Sn-expression, but without significantly affecting hepatic parasite burdens (**Figure 3D** and **Supplementary Figure 4C**).

DISCUSSION

The present study provides evidence for an enhanced infection of *Leishmania* in macrophages that are stimulated with type I interferon. Following experimental triggering with IFN-α, bone marrow-derived macrophages become significantly more susceptible to infection by several laboratory and recent clinical VL strains, linked to enhanced promastigote entry. A putative role for type I IFNs that are typically induced during viral infection is in line with recent studies that document an interplay between leishmaniasis and viral infections. For instance, Ethiopian VL patients co-infected with HIV were found to suffer a higher risk of relapse (36). Adaui and colleagues described a significant association between treatment failure and the presence of an endogenous LRV1 in *L. braziliensis* (6). Another study documented that LRV1 confers an advantage to *L. guyanensis* by promoting survival of infected macrophages through a TLR3/miR-155/Akt signaling pathway (37). These combined literature findings suggest that responses induced by HIV-1 or LRV1 can impact on *Leishmania* infection and efficacy of treatment (38).

Blood transcriptomics conducted in *L. infantum* infected individuals revealed activation of the type I interferon pathway (39), which is also observed in infected BALB/c mice (40). Available literature about the role of type I IFN during leishmaniasis seems to ascribe both protective and exacerbating roles. Protective roles of type I IFNs against *L. donovani* relate to interferon regulatory factor-7 (IRF-7) which plays a critical role in regulating amastigote killing (41). Low doses of IFN-β also conferred iNOS-dependent protection of BALB/c mice from progressive cutaneous and fatal visceral disease caused by low and high doses of *L. major* (42). In addition, the *in vivo* protective effects of CpG-oligodesoxynucleotides against *L. major* depend on IFN-α/β-receptor chain 1 (IFNAR1) signaling (43). *In vitro* exposure of mouse peritoneal macrophages to low doses of IFN-α/β and *L. major* promastigotes leads to the expression of iNOS and subsequent killing of intracellular amastigotes. In contrast, high doses and pretreatment exert antagonistic effects on iNOS induction (44). Type I IFN was also reported to correlate with increased susceptibility to *L. infantum* by triggering IL-27 production by macrophages, resulting in inhibition of Th17 responses (45).

Inspired by the effect of IFN-α and the influence of virus presence, the role of the virus-responsive interferon-stimulated gene sialoadhesin (Sn, CD169, Siglec-1) was explored during *Leishmania* infection as Sn is expressed on macrophages in the major VL target organs. In the liver, KC express Sn and are known to function in clearance of microorganisms and senescent cells/debris from the blood (46). Our data in mice confirm that KCs already express Sn in steady-state conditions. In the spleen, Sn^+ macrophages are a subpopulation of tissue-resident macrophages positioned in the splenic marginal zone that are among the first cell types to encounter invading pathogens (47). These splenic Sn^+ are often referred to as the marginal-zone metallophilic macrophages (46, 48, 49). In the bone marrow, Sn^+ macrophages are found in the stroma (19, 50, 51) and have a scavenging function (19, 20). Importantly, all these Sn^+ macrophage populations become involved during VL infection (52–54). An *in vitro* study further supported a role for sialic acid binding lectins (Siglec-1 and Siglec-5) in *L. donovani* phagocytosis and down-regulation of innate immune signaling responses (3). Next to Sn, other Siglecs were described to be implicated in pathogen-macrophage interactions (3, 55). For *Leishmania* infection, interaction with siglec-5 was shown to deactivate various downstream signaling pathways resulting in a controlled regulation of cytokines in infected macrophages (3).

The present study indicated that the *in vitro* effect of IFN-α is in large part linked to the upregulation of Sn which was confirmed by complementary approaches, i.e., by using

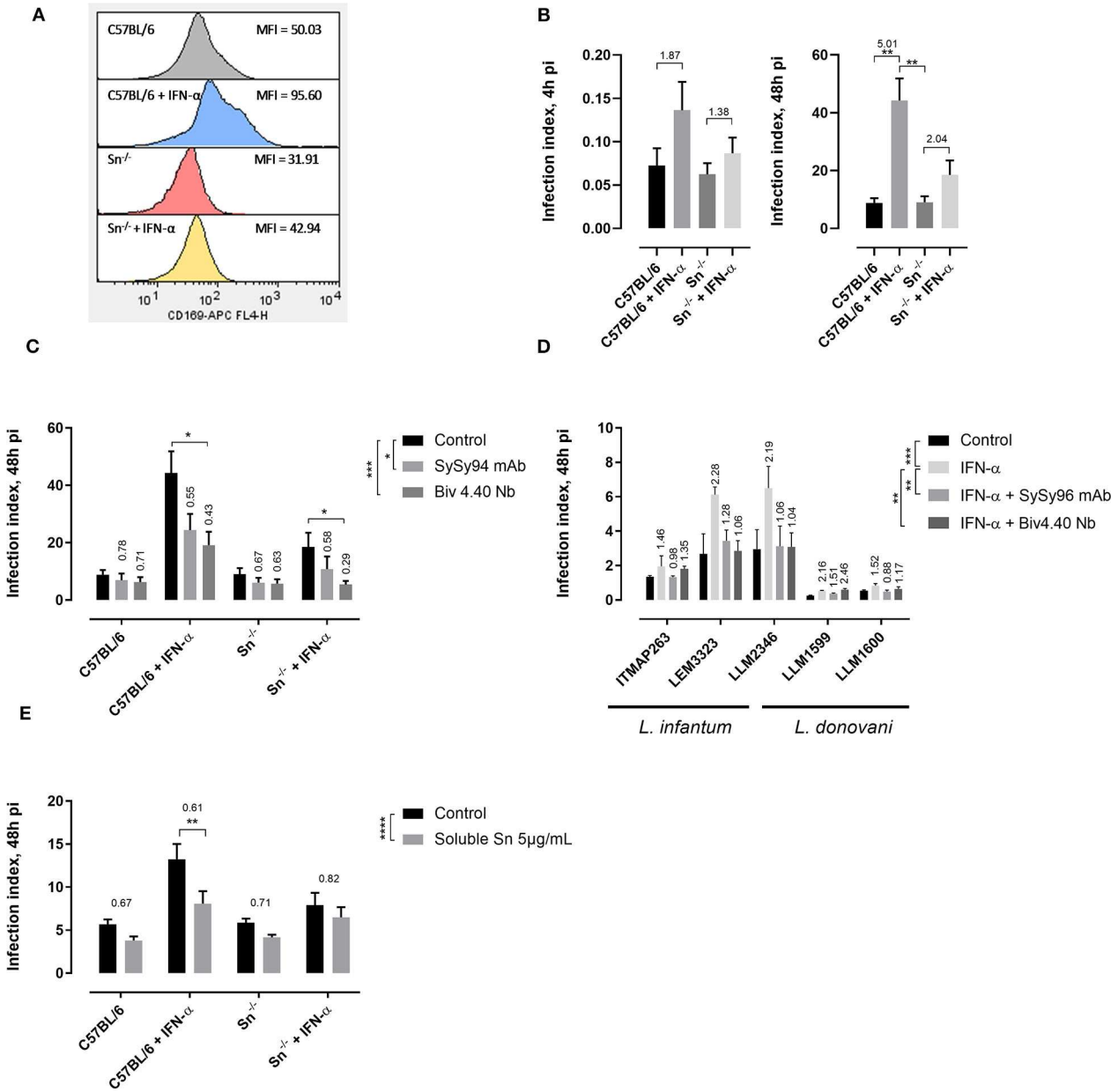

FIGURE 2 | Upregulation of Sn expression by type I interferon enhances *Leishmania* uptake and intracellular multiplication. **(A)** Bone marrow-derived macrophages from C57BL/6 and $Sn^{-/-}$ mice were cultivated for 6 days with or without a 2-day stimulation with 5×10^2 IU/mL IFN-α. Sn surface expression was analyzed by flow cytometry and presented as histograms with the median fluorescence intensity (MFI). These are results from two independent repeats. **(B)** Infection index of LEM3323 promastigote infection (infection ratio 5:1) at 4 and 48 h post-infection in bone marrow-derived macrophages from C57BL/6 and $Sn^{-/-}$ mice either or not pre-stimulated with IFN-α. Results are based on two independent repeats, performed in quadruplicate. The ratio of IFN-α/control is stated above the bars. **(C,D)** Effect of anti-Sn monoclonal antibody SySy94 and anti-Sn Biv4.40 nanobody blocking at a concentration of 10 µg/mL of bone marrow-derived macrophages 1 h prior to infection with **(C)** LEM3323 (infection ratio 5:1) in C57BL/6 mice or **(D)** different *L. infantum* and *L. donovani* strains in BALB/c mice. Results are based on two independent repeats run in triplicate. The ratio of treatment/control is stated above the bars. **(E)** Parasites were incubated with soluble Sn at 5 µg/mL 1 h prior to infection of bone marrow cells. Results were obtained from two independent repeats run in quadruplicate. The ratio of treatment/control is stated above the bars. All results are expressed as mean ± standard error of mean (SEM) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Sn-deficient mice, by treatment of macrophages with either an anti-Sn monoclonal antibody or a novel anti-Sn bivalent nanobody (lacking the Fc-domain) and by pretreating parasites with soluble Sn. Both genetic deficiency and pharmacological inhibition largely counteracted the effect of IFN-α. Similarly, Akiyama et al. found that IFN-α stimulation caused enhanced HIV-1 entry and replication in macrophages that could be reduced by pretreatment with an anti-Sn antibody (16). A

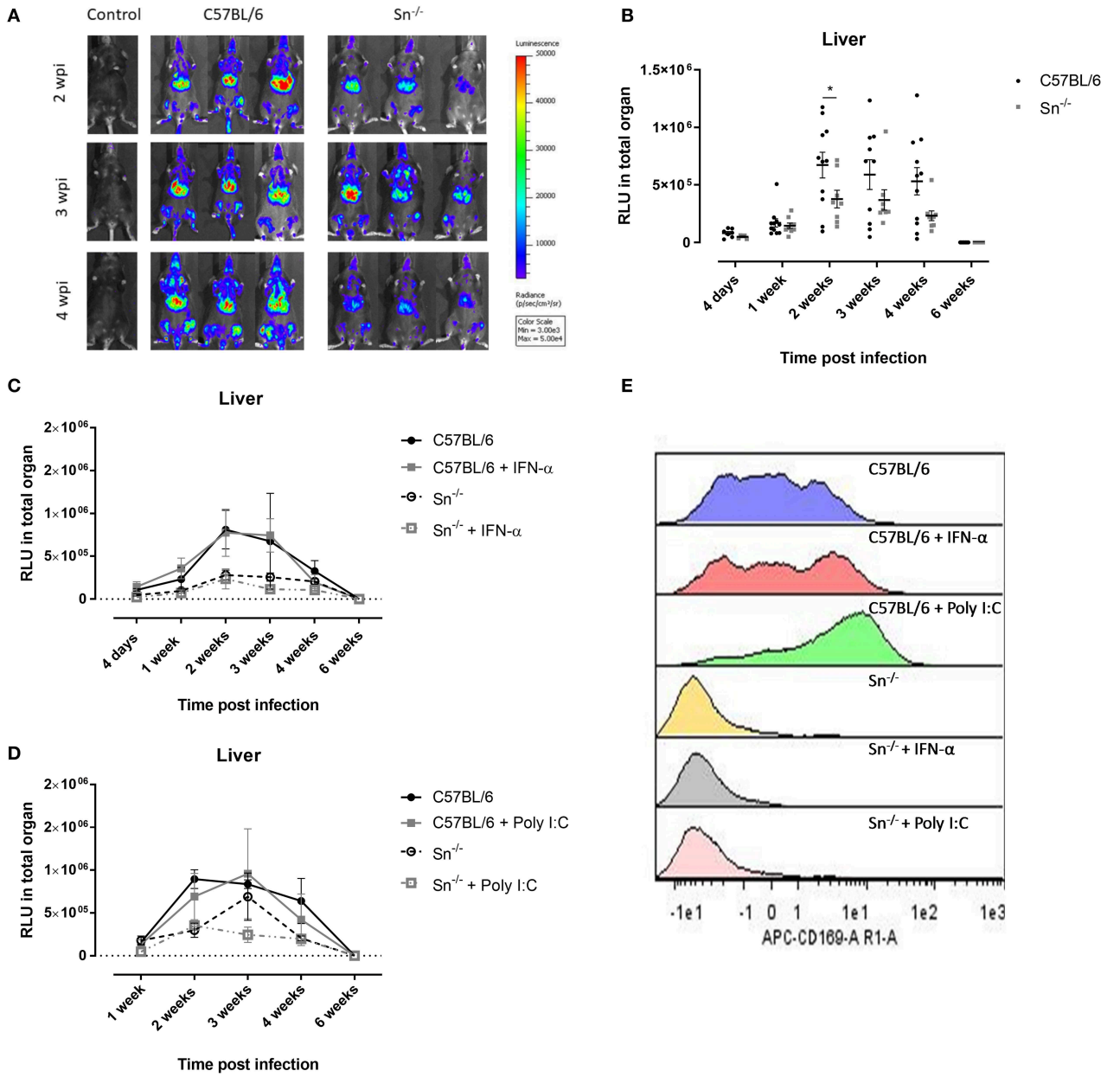

FIGURE 3 | Sialoadhesin plays a moderate role during *in vivo* VL infections. **(A)** Bioluminescent imaging of C57BL/6 and $Sn^{-/-}$ mice infected with *L. infantum* LEM3323^{PpyRE9}. Mice were infected with 1.0×10^8 metacyclic promastigotes in the tail vein. These are representative BLI images of infected wildtype and $Sn^{-/-}$ mice of three independent experiments with $n = 3$ mice/group. **(B)** Relative luminescent units (RLU) in a ROI corresponding to the liver as major target organ. Results are based on three independent experiments with $n = 3$ mice/group (*$p < 0.05$). **(C)** Liver burdens in wildtype and $Sn^{-/-}$ mice either or not subjected to a 3–day subcutaneous pre-exposure to 1,000 IU/g IFN-α. Results in this panel are based on $n = 3$ mice/group. **(D)** Liver burdens in wildtype and $Sn^{-/-}$ mice either or not subjected to intraperitoneal inoculation of 4 μg/g Poly(I:C) 3 days prior to infection, on the day of infection and weekly after infection. Results in this panel are based on $n = 3$ mice/group. Results are expressed as mean ± standard error of mean (SEM). **(E)** Sn expression on KCs in control mice and mice treated with IFN-α or Poly(I:C).

study on porcine primary alveolar macrophages documented a significant reduction of phagocytotic capacity after Sn-blocking with a monoclonal antibody (56). Some effects of IFN-α and the pharmacological inhibitors were also noted in $Sn^{-/-}$ mice. This supports the implication of additional effects of IFN-α and suggests potential compensatory mechanisms in $Sn^{-/-}$ macrophages (e.g., expression of other Siglecs which may explain promiscuity of the pharmacological inhibitors). For instance, IFN-alpha/beta stimulation of macrophages prior to infection was shown to exert antagonistic effects on iNOS expression (44). IFN-α indeed also slightly enhances susceptibility of $Sn^{-/-}$ macrophages. However, these effects are likely to primarily favor

amastigote multiplication, whereas the effects described in this study mainly relate to uptake of the extracellular promastigotes, as illustrated with an established protocol to maximally remove extracellular parasites using horse serum (32).

The role of Sn was further explored *in vivo* by making use of gene-deficient mice (57) and the recently developed bioluminescent *L. infantum* reporter lines LEM3323^{PpyRE9} (29) and ITMAP263^{PpyRE9}. Longitudinal follow-up of hepatic parasite burdens in wildtype and deficient mice revealed that Sn only plays a moderate role during *in vivo* VL infections, depending on the *Leishmania* strain used. These results correspond to earlier findings that the surface display of host sialic acids is strain-dependent, resulting in lower virulence when parasites contain fewer sialic acids (3). We also have shown that Sn is already expressed under steady-state conditions on KCs and that neither IFN-α nor poly (I:C) treatment increase liver burdens.

To our knowledge, this is the first study that explored the *in vivo* contribution of Sn to *Leishmania* infection. The role of Sn seems to vary substantially depending on the pathogen involved. Two studies on *Plasmodium* demonstrated that mice depleted of Sn^+ macrophages developed significantly higher parasitaemia, weight loss and mortality relative to controls (58, 59), indicating that Sn^+ macrophages are effective in controlling *Plasmodium in vivo*. *Streptococcus pneumonia*, known to replicate inside Sn^+ splenic macrophages, was not hampered in Sn-deficient mice (60). A study by Martinez-Picado (61) investigated the outcome of HIV-1 in Sn null individuals and found no measurable impact of a truncation in the Sn protein on HIV-1 acquisition or AIDS outcome *in vivo*. This actually contrasts with the *in vitro* functional role of Sn in HIV-1 *trans*-infection. Similarly, our study found a definite functional role *in vitro* but a rather moderate impact *in vivo*. A possible explanation is that Sn primarily affects early infection with promastigotes, but plays a less prominent role during an established infection with amastigotes. The amastigote/promastigote-comparison in our study was limited to the ITMAP263 strain which is not highly modulated by IFN-α but for which hamster spleen-derived amastigotes were available. This could not show elevated uptake and expansion of *L. infantum* ITMAP263 amastigotes in IFN-α stimulated macrophages with elevated surface Sn-expression levels. Amastigotes may indeed employ different strategies for macrophage entry than promastigotes (62), especially because sialic acids on the *Leishmania* surface are acquired by adsorption of serum proteins from the host (23, 24). A study by Chava and colleagues (63) using the Indian *L. donovani* strain MHOM/IN/83/AG83 did demonstrate the presence of sialoglycoconjugates on the amastigote surface, which suggests that strain-dependent differences can be expected.

A recent paper delivered a proof-of-concept that blocking Sn by monoclonal antibodies could be of therapeutic value as exemplified by the halting of Ebola viral uptake and cytoplasmic entry in dendritic cells (64). The fact that Sn null individuals exist also suggests that Sn may serve as a safe therapeutic target (61). Our data on VL indicate that Sn plays a moderate role *in vivo* depending on the parasite strain, although *in vitro* data suggest significant exacerbation in particular conditions with presence of type I interferon. It remains to be seen if the *in vivo* role of the IFN-α/Sn axis could be more prominent under certain conditions, determining whether therapeutic targeting of Sn may deserve further exploration during VL infection and/or in combination with drug treatment.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical committee of the University of Antwerp, Belgium.

AUTHOR CONTRIBUTIONS

LV, PD, LM, and GC: conceived and designed the experiments. LV, DB, MV, LD, DM, and SH: performed the experiments. LV, DB, and GC: analyzed the data. LV, and GC: wrote the manuscript. PD, LM, and GC: critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01113/full#supplementary-material

Supplementary Figure 1 | Purification and binding of Biv4.40 nanobody to Sn-expressing CHO cells. **(A)** Size exclusion chromatography (SEC) profile of Biv4.40 (30 kDa) with an elution peak at 22 min. Abs, absorbance at 280 nm. Biv4.40 binding onto CHO control cells **(B)** and CHO cells expressing mouse

sialoadhesin (mSn$^+$) **(C)** Cells were stained with Biv4.40 nanobody and anti-myc Alexa Fluor 488 (green) and with DAPI (blue).

Supplementary Figure 2 | Gating strategy for KCs. KCs were gated according to their CD45$^+$ F4/80$^+$ Tim4$^+$ expression profile.

Supplementary Figure 3 | Cytokine profile over time after infection with LEM3323^{PpyRE9} in wildtype and Sn$^{-/-}$ C57BL/6 mice. Cytokine data of serum samples from C57BL/6 and Sn$^{-/-}$ mice infected in the tail vein with 1.0 × 108 metacyclic promastigotes of *L. infantum* MHOM/FR/96/LEM^{3323PpyRE9}. Cytokines **(A)** IL-6, **(B)** IL-10, **(C)** TNF-γ, **(D)** KC/GRO and **(E)** IFN-γ were analyzed with multiplex ELISA. Results in this panel are based two experiments with $n = 3$ mice/group. Results are expressed as mean ± standard error of mean (SEM).

Supplementary Figure 4 | Role of Sn during ITMAP263^{PpyRE9} infection. **(A)** Bioluminescent imaging of C57BL/6 and Sn$^{-/-}$ mice infected with *L. infantum* ITMAP^{PpYRE9}. Mice were infected with 1.0 × 10^8 metacyclic promastigotes in the tail vein. **(B)** Relative luminescent units (RLU) in a ROI corresponding to the liver as major target organ. **(C)** Liver burdens in wildtype and Sn$^{-/-}$ mice either or not subjected to intraperitoneal inoculation of 4 μg/g Poly(I:C). Results are expressed as mean ± standard error of mean (SEM).

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Leishmania amazonensis Subverts the Transcription Factor Landscape in Dendritic Cells to Avoid Inflammasome Activation and Stall Maturation

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Leishmania parasites are the causative agents of human leishmaniases. They infect professional phagocytes of their mammalian hosts, including dendritic cells (DCs) that are essential for the initiation of adaptive immune responses. These immune functions strictly depend on the DC's capacity to differentiate from immature, antigen-capturing cells to mature, antigen-presenting cells—a process accompanied by profound changes in cellular phenotype and expression profile. Only little is known on how intracellular *Leishmania* affects this important process and DC transcriptional regulation. Here, we investigate these important open questions analyzing phenotypic, cytokine profile and transcriptomic changes in murine, immature bone marrow-derived DCs (iBMDCs) infected with antibody-opsonized and non-opsonized *Leishmania amazonensis* (*L.am*) amastigotes. DCs infected by non-opsonized amastigotes remained phenotypically immature whereas those infected by opsonized parasites displayed a semi-mature phenotype. The low frequency of infected DCs in culture led us to use *Ds*Red2-transgenic parasites allowing for the enrichment of infected BMDCs by FACS. Sorted infected DCs were then subjected to transcriptomic analyses using Affymetrix GeneChip technology. Independent of parasite opsonization, *Leishmania* infection induced expression of genes related to key DC processes involved in MHC Class I-restricted antigen presentation and alternative NF-κB activation. DCs infected by non-opsonized parasites maintained an immature phenotype and showed a small but significant down-regulation of gene expression related to pro-inflammatory TLR signaling, the canonical NF-kB pathway and the NLRP3 inflammasome. This transcriptomic profile was further enhanced in

DCs infected with opsonized parasites that displayed a semi-mature phenotype despite absence of inflammasome activation. This paradoxical DC phenotype represents a *Leishmania*-specific signature, which to our knowledge has not been observed with other opsonized infectious agents. In conclusion, systems-analyses of our transcriptomics data uncovered important and previously unappreciated changes in the DC transcription factor landscape, thus revealing a novel *Leishmania* immune subversion strategy directly acting on transcriptional control of gene expression. Our data raise important questions on the dynamic and reciprocal interplay between *trans*-acting and epigenetic regulators in establishing permissive conditions for intracellular *Leishmania* infection and polarization of the immune response.

Keywords: dendritic cell, *Leishmania amazonensis,* amastigote, transcription factor, NF-κB, NLRP3, transcriptome, cell sorting

INTRODUCTION

Dendritic cells (DCs) are essential components of the immune system initiating antigen-specific adaptive immune responses to foreign antigens and maintaining tolerance to self-antigens (1). They are recognized as key actors of the immune response to infection caused by viral, bacterial, and eukaryotic pathogens (2). Many of these infectious agents have evolved strategies to interfere with DC immune functions promoting their own survival. One strategy is represented by intracellular DC infection, which allows the pathogen to hide from immune recognition and to subvert DC signaling, gene expression, and immune activation. This is very well illustrated for the protozoan parasite *Leishmania amazonensis (L. am)* (3–5), one of the causative agents of diffuse cutaneous Leishmaniasis in South America (6, 7). Upon infection, DCs display reduced activation, maturation, *in vivo* and *in vitro* antigen-presenting capacities, and migration properties (8–11). These alterations were linked to the subversion of key signaling kinases, including STAT1/2/3 and ERK1/2 (10–14).

A number of pattern recognition receptors, including NOD-like receptors are involved in key steps of DC maturation and migration (15). NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) is an intracellular sensor that is synthesized in response to a "priming signal" involving the engagement of cytokine or Toll-like receptors and further activated by pathogen- or damage-associated molecular patterns (PAMPs/DAMPs) such as ATP. This process triggers caspase-1 activation, which cleaves pro-IL-1β and pro-IL-18 into mature cytokines further secreted during the adaptive immune response (16, 17). While IL-1β favors efficient protective T cell responses (16, 18), notably Th17-mediated immunity (17, 19, 20), IL-18 potentiates IL-12-dependent development of IFN-γ-producing Th1 cells (17, 21). In DCs and macrophages, NLRP3 is activated in response to bacteria (22–24), fungi (25, 26), viruses (27, 28), and certain parasites (29). Recent studies evaluated NLRP3 activation in *Leishmania*-infected macrophages *in vitro* and *in vivo* (30, 31). While a previous study showed that *L. am* promastigotes caused NLRP3 activation in infected tissues *in vivo* (31), our recent study revealed that *L. am* amastigotes did not activate the inflammasome, neither *in vitro* in bone marrow-derived macrophages nor *in vivo* in lesional macrophages (30).

In contrast to macrophages, no information is available on the status of NLRP3 inflammasome activation and cell maturation in *L. am*-infected DCs, despite their essential roles in immune priming during *Leishmania* infection (32). Here we investigated these important open questions using primary, bone marrow-derived DCs (BMDCs) infected with *Ds*Red*2*-transgenic parasites that allowed for FACS-purification of infected cells (33). We thus overcame one of the major challenges in systems-level analysis of *Leishmania*-infected DCs represented by the low *in vitro* infection level (4, 12, 34, 35), which dilutes biological signals due to the presence of uninfected DCs (33). Transcriptomic analyses of sorted DCs using the Affymetrix GeneChip technology revealed that *L. am* infection causes important changes to the host cell transcription factor landscape that correlated with transcriptional activation of the alternative NF-κB pathway, but inhibition of the canonical NF-κB pathway as well as DC maturation and inflammasome activation.

MATERIALS AND METHODS

Mice

Female BALB/c mice and Swiss *nu/nu* mice were purchased from Charles River (Saint Germain-sur-l'Arbresle, France). Female *Fcer1g* knockout (BALB/cByJMTac-*Fcer1g*tm1 N12) and corresponding wild type mice were purchased from Taconic (Taconic Biosciences, Inc.).

Parasites, Bacteria, and Cell Lines

L. amazonensis strain LV79 (MPRO/BR/1972/M1841) genetically modified to stably express fluorescent *Ds*Red2 (33) were propagated in Swiss nu/nu mice. *L. amazonensis* amastigotes were isolated 2 months after infection from mouse footpad lesions purified as described (36). These amastigotes did not present antibodies at their surface (12). *Mycobacterium bovis* BCG (bacillus Calmette Guérin) was grown in Sauton medium, recovered as previously described (37) and stored at −80°C until use. The J558 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal

calf serum (FCS; Dutscher, Brumath, France) to get GM-CSF rich supernatants.

DC Culture and Infection by *L. amazonensis* Amastigotes

DCs were differentiated from bone marrow cells of 6-week-old wild type BALB/c or BALB/cByJMTac-*Fcer1g*tm1 N12 mice (BMDCs) (12). Briefly, bone marrow cells were seeded at 2 $\times$ 10^6 cells per 100 mm diameter bacteriological grade Petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in 10 ml of Iscove's modified Dulbecco's medium (IMDM; BioWhittaker Europe, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; Dutscher, Brumath, France), 1.5% supernatant from the GM-CSF producing J558 cell line, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and 2 mM glutamine. Cultures were incubated at 37°C in a humidified atmosphere with 7% CO_2. On day 6, suspended cells and loosely adherent cells were harvested using 1% EDTA (Versene) in PBS without Ca^{2+} and Mg^{2+} and cultured in the same plastic ware in complete IMDM supplemented with 10% of the primary culture supernatant. On day 10, cells were harvested following EDTA treatment as below and distributed in hydrophobic 6-well-plates (Greiner, St. Marcel, France) at a concentration of 9 $\times$ 10^5 cells/ml in 3 ml of complete IMDM.

On day 14, freshly isolated *Ds*Red2-LV79 amastigotes or live BCG were incubated with BMDCs at ratios of 4:1 and 10:1, respectively. Amastigotes were opsonized or not with heat-inactivated immune serum prepared from *L. am*-infected BALB/c mice for 1 h at 4°C. Serum was removed by two washing steps in PBS (1,200 g, 10 min, 4°C). DC cultures were placed at 34°C for 24 h.

Dendritic Cell Phenotyping by Flow Cytometry

BMDCs were resuspended in cold Dulbecco's PBS with 2% FCS and 0.05% sodium azide (PBS-FCS-Az) and transferred to a round-bottomed 96-well-plate (Costar, Corning, FR) at a concentration of 2–5 $\times$ 10^5 cells/well. All subsequent steps were carried out on ice. Cells were incubated in PBS-FCS-Az supplemented with 10% donkey serum for 20 min. After centrifugation, DCs were incubated for 30 min in PBS-FCS-Az containing the primary biotinylated Abs: 2D7 (anti-CD11a/LFA-1 α-chain), M1/70 (anti-CD11b/CR3 α-chain), HL3 (anti-CD11c/p150, 95 α-chain), M1/69 (anti-CD24/HSA), 3/23 (anti-CD40), 3E2 (anti-CD54/ICAM-1), 16-10A1 (anti-CD80/B7-1), GL1 (anti-CD86/B7-2), 2G9 (anti-I-Ad/IEd) BD bioscience (San Diego, CA). Biotin-labeled IgGs, used as isotype controls, were obtained from BD bioscience and Caltag Laboratories (San Francisco, CA). After three washing steps, they were incubated with PBS-FCS-Az containing phycoerythrin-conjugated streptavidin for 30 min, cells were washed and treated with the CytoFix/CytoPerm reagent (BD bioscience) for 30 min. For dual staining including parasite detection, cells were also incubated either with the amastigote-specific 2A3-26 mAb (38) followed by fluorescein isothiocyanate-conjugated donkey anti-mouse Ig F(ab′)2 fragments or with the Alexa Fluor 488-conjugated 2A3-26 mAb to allow the detection of intracellular *Leishmania*. All washing and incubation steps were performed with Perm/Wash buffer (BD bioscience) supplemented with 10% donkey serum. Appropriate isotype controls (irrelevant rat, mouse, or hamster mAbs) were used at the same concentrations than those used for primary Abs. Flow cytometry results were acquired using a LSR Fortezza™ cytometer (Becton Dickinson, Mountain View, CA).

Dendritic Cell Phenotyping by Fluorescence Microscopy

For epifluorescence and confocal microscopy analysis, DCs were collected by EDTA treatment, centrifuged, and resuspended in Dulbecco's PBS without $Ca2^+$ and $Mg2^+$. DCs were centrifuged onto poly-L-lysine-coated glass coverslips and incubated at 34°C for 30 min, before fixation with paraformaldehyde, permeabilization with saponin, and immunostaining (12). Cell preparations were mounted in Mowiol (Calbiochem, San Diego, CA) before analysis using an Axiophot Zeiss epifluorescence or a LSM 510 Zeiss confocal microscope. Confocal microscopy images were acquired and analyzed using the LSM 510 software (version 3.1).

Preparation of DC Samples for High Speed Cell Sorting

After 5 min of contact with the Versen-EDTA solution at 34°C, DCs were carefully detached, resuspended at 4°C in Dulbecco's PBS with 2% FCS (PBS-FCS) and transferred to a 15 ml tube (Falcon; BD Biosciences, San Jose CA) at a concentration of 6 $\times$ 10^6 cells/ml. Cells were centrifuged (300 g, 5 min, 4°C), and resuspended in PBS-FCS supplemented with 10% heat-inactivated donkey serum for 5 min. Cells were then incubated for 30 min in PBS-FCS containing 0.2 µg/ml of the anti-MHC class II monoclonal antibody (mAb) (M5/114) or the corresponding IgG2a isotype control mAb, both conjugated to PE-Cy5 (eBioscience). After two washes, cells were resuspended at 5 $\times$ 10^6 cells/ml in PBS containing 3% FCS and 1% J558 supernatant. Cell aggregates were dissociated using a 70-µm filter (Falcon) and placed on ice until cell sorting.

Cell Sorting

Once stained with the M5/114 mAb as described above, live DCs were sorted using a FACSAria (BD Biosciences) equipped with sealed sample injection and sort collection chambers that operate under negative pressure, and operated by the BD FACSDiva™ software (BD Biosciences). FSC and SSC parameters were displayed on a linear scale and used to discard cell debris. To avoid the sorting of cell doublets or cell aggregates, single cells were sequentially selected on SSC-H/SSC-W, and FSC-H/FSC-W dot plots. Infected DCs were sorted by selecting cells expressing surface MHC Class II molecules and containing *Ds*Red2 expressing intracellular amastigotes (576/26 bandpass filter). Uninfected DCs were sorted on the basis of MHC Class II expression using the same gating procedure as for infected DCs. Sorting conditions included: (i) sheath pressure of 70 Psi, (ii) flow

rate of 7, and (iii) 70 μm nozzle tip. Cells were collected at 4°C in polypropylene tubes (BD Biosciences) previously coated with FCS (overnight incubation at 4°C). Sorted cells were immediately used for RNA isolation. All these experimental procedures were performed according to biosafety level two practices (BSL2) (39).

RNA Integrity Quality Control

RNA isolation was performed with the RNeasy^{+} isolation kit (Qiagen) according to the manufacturer's instructions. Evaluation of RNA quality was carried out by optical density measurement using a Nanodrop device (Kisker, http://www.kisker-biotech.com) and by electrophoresis on a Lab-on-a-chip product using the Agilent 2100 Bioanalyzer (Agilent, http://www.chem.agilent.com). RNA Integrity Number (RIN) scores were monitored for each sample providing an objective and standardized measure of RNA quality on a scale of 1–10 (the value 10 corresponding to the highest quality) (40).

Transcriptomic Analysis

RNA samples were subjected to GeneChip hybridization on the Mouse Genome 430_2.0 Array (Thermo Fisher Scientific) following the Affymetrix two-cycle labeling protocol. Affymetrix MIAME-compliant data have been made available through Gene Expression Omnibus databases (www.ncbi.nlm.nih.gov/projects/geo/, accession: GSE144039). Data processing, background correction, normalization, and signal quantification were carried out using RMA algorithm using the Bioconductor "affy" package version 1.62.0 (41). Differential expression was determined using R version 3.6.1 and the Bioconductor limma package version 3.40.6 (42). Raw *p*-values were adjusted for multiple testing using the Benjamini and Hochberg algorithm, and probesets with an adjusted *p*-value lower than 5% were considered differentially expressed.

Gene Ontology Analysis

Raw probe-set identifiers were translated into ENTREZ prior to the enrichment analysis since gene-sets are defined by lists of ENTREZ gene identifiers. Additionally, ENTREZ genes linked to several probe-sets were associated with the probe-set having the most variable expression across replicates. Functional gene-set enrichment analysis was performed using the Fisher statistical test for the over-representation of differentially expressed genes (adjusted *P*-value lower than 5%). The genes lists were used to interrogate the gene-set collection of Gene Ontology (GO) annotations selected from the Molecular Signatures Database MSigDB v6.2 (43). Only gene-sets with an FDR lower than 0.05 were considered significantly enriched in differentially expressed genes.

Network Analysis

Networks shown in **Figure 7** were derived from the String Database (https://string-db.org) with text mining, neighborhood, experiments, gene fusion, databases, co-expression, and co-occurrence as active interaction sources. Network representation was performed with Cytoscape (V3.7.1). Network edges correspond to active interactions and are depicted as gray lines, whereas black arrows correspond to molecule interactions verified in the literature. The list of transcription factors, their co-regulators and epigenetic factors was created manually from different web sources (AnimalTFDB/OMICS_01856, Riken Transcription Factor Database, TRRUST v2, and mTFkb) (44–46) (**Supplementary Table 1**).

Western Blotting

DCs were lysed in RIPA buffer (R0278, SIGMA) supplemented with a cocktail of anti-proteases and anti-phosphatases inhibitors (MS-SAFE, SIGMA). Proteins were resolved by SDS–PAGE on NuPAGE gels (4–12% Bis-Tris) in MOPS buffer and electroblotted onto polyvinylidene difluoride (PVDF) membranes in transfer buffer. Membranes were blocked with 5% fat-free milk in 1×Tris-buffered saline containing 0.25% Tween 20 and then probed overnight at 4°C with the following primary antibodies: anti-NLRP3 (MAB7578, R&D Systems), anti-OPTINEURIN (polyclonal rabbit antiserum against amino-acids 84-164 of OPTN) (47), anti-IRAK1 (H-273, sc-7883, Santa Cruz), anti-MYD88 (ab2064, Abcam), anti-RELA (ab32536, Abcam) and anti-RELB (ab#1319 from Nancy Rice) (48), and anti-β-ACTIN (4970, Cell Signaling) antibodies.

Following incubation with the appropriate peroxydase conjugate secondary antibodies, membranes were revealed by SuperSignal West Pico reagent (ThermoFisher Scientific) in a high-resolution PXi machine (Syngene). Relative protein expression was calculated by densitometric analysis using the ImageJ software. The integrated density was measured on scanned gels with inverted images free of pixel saturation using a region of interest for each specific band. For every band, the ratio between the values obtained for the target protein and the β-actin normalization control was calculated and fold changes calculated using the control sample as calibrator, control values of uninfected and unstimulated samples being set to 1.

Cytokine/Chemokine Profiling and Quantitation in Culture Supernatants

Cytokine/chemokine profiling was performed by the mouse XL cytokine array kit (R&D Systems) according to the manufacturer's instructions. Membranes were revealed by SuperSignal West Pico reagent (ThermoFisher Scientific) in a high-resolution PXi machine (Syngene). Semi quantitative analysis was performed with the Quickly & Easily Process Proteome Profiler^{TM} Antibody Arrays software (R&D Systems) on the membrane scans. IL-1β quantification in the supernatants was performed by using the mouse instant ELISA kit (eBioscience) following the manufacturer's recommendations.

Statistical Analyses

Two-sided Student's paired *t*-tests were used to compare data from flow cytometry experiments and gene expression studies performed on sorted samples ($6 < n < 13$). A non-parametric Mann-Whitney bilateral *U*-test was used for gene expression comparisons on unsorted samples ($n = 5$).

RESULTS

L. amazonensis Amastigotes Stall DC Maturation

We first assessed the maturation level of DCs in response to infection with lesion-derived *L. amazonensis* amastigotes (*L.am*). BMDCs were incubated with parasites without prior opsonization or were opsonized with immune serum obtained from *L. am*-infected BALB/c mice (*L.am*-IS), known to favor *Leishmania* uptake through Fcγ receptors and to facilitate the acquisition of protective immunity (12, 49). To evaluate the extent of DC maturation after 24 h of infection, we first stained DCs for MHC Class II molecules and the peptide-loading facilitator H2-M, which co-localize in immature but dissociate in mature DCs (50) (**Figures 1A1,2**, **Supplementary Figure 1**). Epifluorescence microscopy analyses revealed that these markers predominantly co-localize in discrete vesicles in the majority (80%) of DCs infected with non-opsonized amastigotes similar to uninfected control, but dissociate during infection with Ab-opsonized amastigotes (>70% of infected cells) as observed for BCG-treated, mature DCs (**Figures 1A1–3**).

We next quantified surface expression of costimulatory (CD80, CD86, and CD40) and adhesion (CD24 and CD54) molecules diagnostic for DC maturation using flow cytometry. As expected, BCG-infection strongly increased the surface expression of all markers compared to control (**Figure 1B**). Conversely, no significant increase in marker expression was observed in response to infection with non-opsonized *L. am*, thus confirming their stealthy entry (**Figure 1B**). Surprisingly, surface marker expression was only slightly increased in DCs infected with Ab-opsonized *L. am*, a feature however clearly dependent upon the presence of the Fcγ chain as no phenotypic cell surface modulation could be evidenced in $Fc\gamma^{-/-}$ BMDCs infected with opsonized *L. am* parasites (**Supplementary Figure 2**). These slight changes observed at the DC surface were also contrasting with the clear maturation signal revealed by MHC Class II/H2-M dissociation (**Figure 1A**). This discrepancy indicates a stalled maturation process that was confirmed by the absence or lower secretion levels of a series of chemokines (CCL2, CCL3, CCL5, CCL12, LIX/CXCL5) and cytokines (TNF, IL6, IL1α, IL12p40, and IL1RA) in *Leishmania*-infected samples compared to BCG-infected DC cultures (**Figures 1C1**, **2**, **Supplementary Figure 3**). These results further demonstrate that in contrast to BMDC infection *in vitro* with *L. braziliensis* promastigotes, *L. amazonensis* amastigotes do not induce TNF in non-infected bystander cells (51). Together these data reveal an important role of *Leishmania* opsonization in shaping the DC response, and uncover a novel parasite immune-subversion strategy to stall the DC maturation process.

L. amazonensis Avoids Inflammasome Activation in DCs

The NOD-like receptor protein NLRP3 is a cytosolic sensor that triggers maturation and secretion of pro-inflammatory IL-1β (52). Contrary to the LPS/ATP-treated positive control, infection with Ab-opsonized or non-opsonized parasites does not result in IL-1β secretion (**Figure 2A1**), demonstrating the absence of inflammasome activation as we observed previously in macrophages (30). DCs infected with Ab-opsonized amastigotes even showed a significant reduction of NLRP3 expression (**Figure 2A2**), which correlated with increased expression of Optineurin (OPTN), an inhibitor of the TLR-induced, canonical NF-κB pathway. In contrast, infection neither affected expression of key activators of the TLR pathway (IRAK1, MYD88, and RELA) nor RELB, a key element pivotal for DC differentiation, maturation, and MHC Class I-restricted presentation (53, 54) (**Figure 2B**). Our data thus identify induction of OPTN expression as a potential key mechanism of *Leishmania* to escape detection by the DC TLR–NF-κB–NLRP3 innate immune axis.

Transcriptome Profiling of *L. amazonensis*-Infected DCs

We next performed transcriptomic analyses on *Leishmania* infected DCs to gain further insight into the mechanisms underlying their stalled maturation. A major challenge in the systems-level analysis of DC/*Leishmania* interaction is to avoid the dilution of any infection-related signal in the analysis of heterogeneous DC populations with low percentage of *Leishmania*-hosting DCs (33). Indeed, we observed an infection efficiency of 10.8 ± 1.8 and 19.5 ± 2.5 % using non-opsonized and Ab-opsonized amastigotes, respectively. To overcome this challenge, we used *Ds*Red2 transgenic parasites to purify infected DCs by high-speed cell sorting as previously designed and validated (33). This approach was based on a bi-parametric analysis and sorting, using MHC Class II expression as a common marker for sorting control and infected DCs, and *Ds*Red2-fluorescence as a marker for intracellular infection (MHC Class II positive, *Ds*Red2 positive DCs, see **Figure 3A**, *L. am* and *L. am*-IS conditions). Total RNA was isolated from purified DCs in three independent, biological experiments, tested for quality (**Supplementary Figure 4A**) and processed for probe-set hybridization and quantification by the Affymetrix microarray technology.

Cluster analysis of modulated probe-sets revealed highly reproducible changes in the DC transcript profiles in response to non-opsonized and opsonized amastigotes, with respectively 2,077 and 3,293 genes showing differential expression compared to uninfected samples ($p < 0.05$, $|FC| > 1.5$, **Figure 3B**, **Supplementary Figure 4B**). 74.1 and 70.95% of these regulated genes were down modulated in DCs infected by non-opsonized and Ab-opsonized amastigotes, respectively. Opsonization generally enhanced the expression changes observed in DCs infected with non-opsonized parasites (**Figure 3C**, green dots, and **Supplementary Figure 4B**). Finally, gene set enrichment analysis revealed that non-opsonized and Ab-opsonized amastigotes interfered with numerous DC processes including those implicated in the regulation of the MHC Class II protein complex, peptide antigen binding, and cell adhesion (**Figure 3D** and **Supplementary Table 2**). Interestingly, as judged by the decrease in transcript abundance, DC infection with Ab-opsonized amastigotes interfered with various immune-related processes,

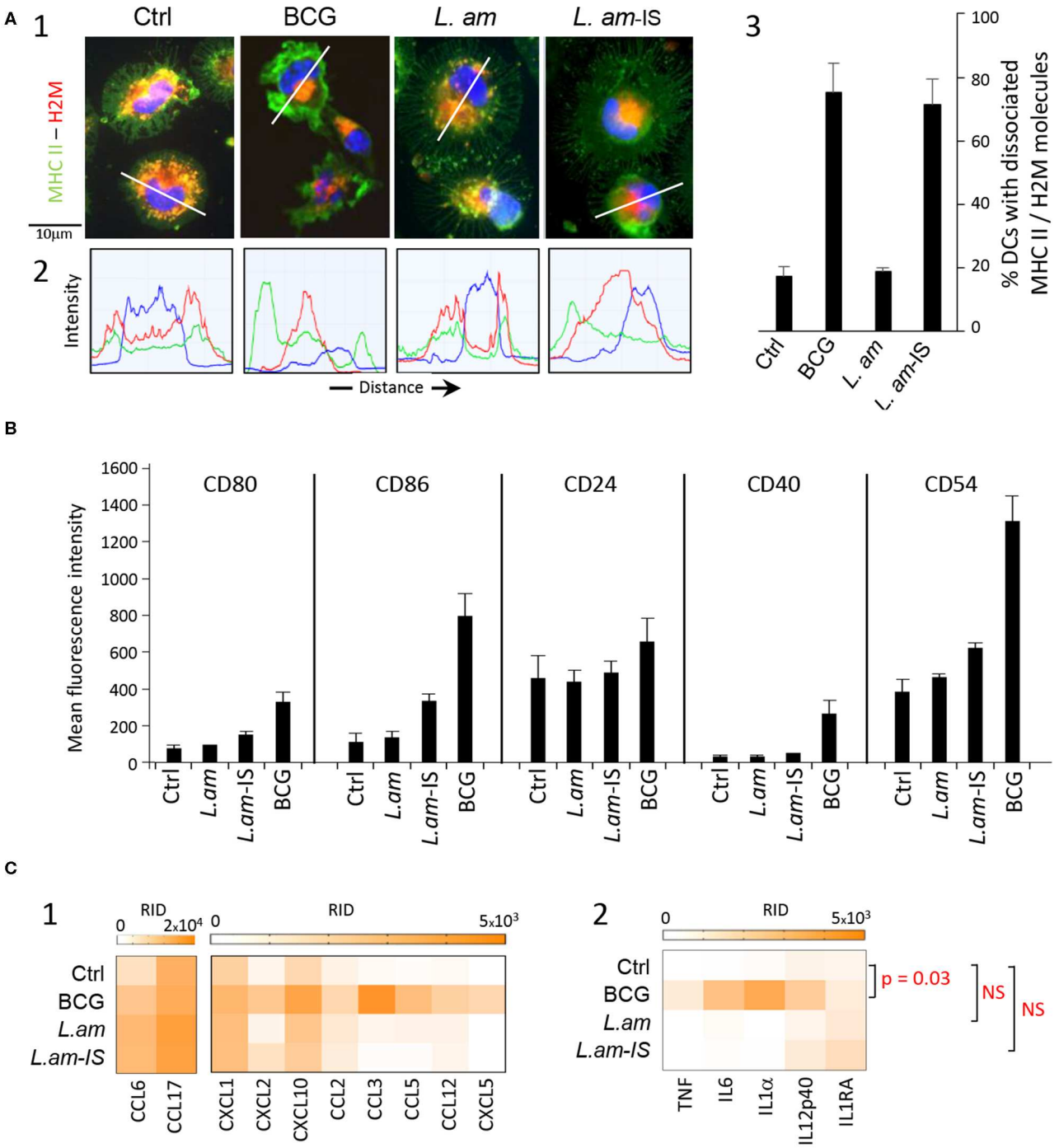

FIGURE 1 | Multiparametric characterization of dendritic cell cultures. BALB/c-derived BMDCs alone (Ctrl) or incubated for 24 h with BCG or *L. amazonensis* amastigotes without (*L. am*) or with Ab-epsonization (*L. am*-IS) were analyzed. **(A)** Confocal microscopy imaging of DCs stained for MHC Class II and H2-M molecules. Images of representative DCs from the different conditions are displayed. (1) Merged images of the three detection channels showing MHC Class II molecules (green), H2-M molecules (red), and nuclear DNA (blue). (2) Intensity distribution of the three detected fluorescence signals along the transect line indicated in (1). (3) Percentage of DCs showing dissociated localization for MHC Class II and H-2M ($n = 3$ independent experiments). **(B)** Expression levels of co-stimulatory and adhesion molecules. Flow cytometric analysis was performed on DCs from the different culture conditions ($n = 3$–6 independent experiments). Mean (+ SD) fluorescence intensity (MFI) signals obtained for the corresponding stainings are shown. **(C)** Chemokine and cytokine determination in culture supernatants. The level of secretion was analyzed at 24 h post-infection in culture supernatants using the proteome profiler mouse XL cytokine array kit. Heat maps of background-corrected Raw Integrated Density (RID) values for technical duplicates are represented for chemokines (1) and cytokines (2). Scanned membranes of protein arrays are shown in **Supplementary Figure 1**. *P*-values for inter-group differences are indicated. NS, not significant.

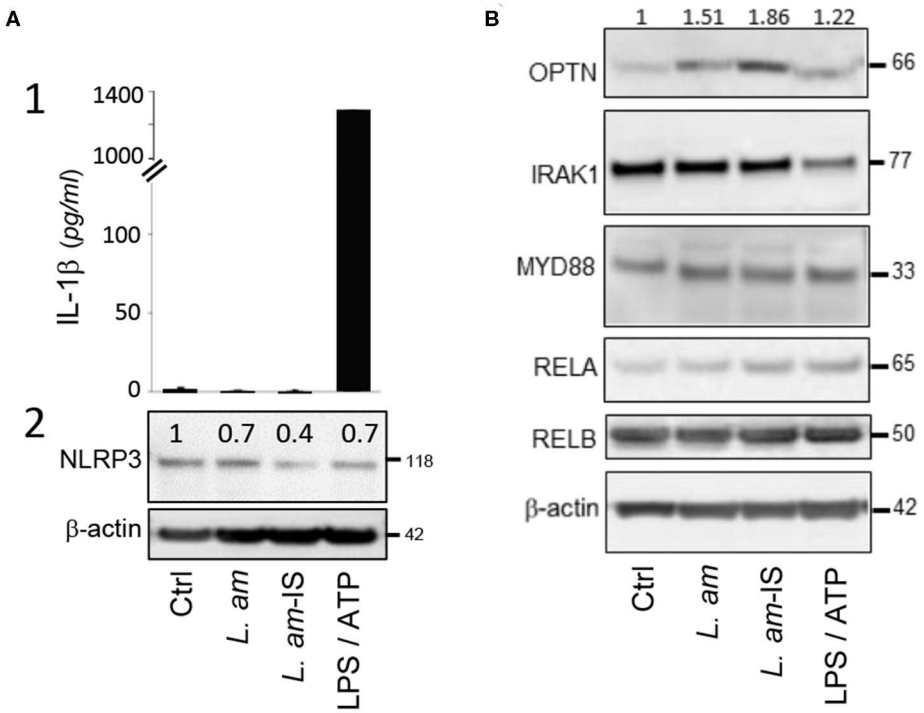

FIGURE 2 | Analysis of protein abundance of key components of the TLR – NF-kB – NLRP3 axis. BMDCs alone (Ctrl), treated with LPS/ATP for inflammasome activation, or incubated for 24 h with either BCG or *L. amazonensis* amastigotes without (*L. am*) or with Ab-opsonized (*L. am*-IS) were analyzed. **(A)** Analysis of DC inflammasome priming and activation. The status of inflammasome priming and activation was analyzed following IL-1β secretion into culture supernatants and quantifying NLRP3 expression in cell lysates. (1) IL-1β was quantified by ELISA and data displayed as the mean quantity of IL-1β ± SEM (technical duplicates of one representative experiment, $n = 2$ independent experiments). (2) NLRP3 detection by Western Blotting. Values shown on top of the lanes indicate the relative abundance of the normalized value of NLRP3 to β-actin compared to control DCs (Ctrl). **(B)** Protein expression of key members of the TLR-NF-κB signaling pathway. The abundance of OPTN, IRAK1, MYD88, RELA, and RELB in cell lysates was analyzed by Western Blotting. Values shown on top of the lanes indicate the relative abundance of the normalized value of OPTN protein to β-actin compared to control DCs (Ctrl) for a representative Western Blot ($n = 2$ independent experiments).

including NF-κB signaling and the production of IL-1β, IL-12, and eicosanoids.

L. amazonensis Infection Affects Genes Linked To DC Maturation and Pro-inflammatory Response

The fold changes observed in RNA abundance between infected and control DCs were calculated and visualized for genes coding for co-stimulatory molecules, key DC markers, Fcγ receptors and MHC molecules (**Figure 4A**) as well as cytokines, chemokines and their receptors (**Figure 4B)**. Overall, expression changes observed during amastigote infection were generally enhanced by opsonization, with a mean |linear FC| = 2.116 ± 0.1004 and 3.138 ± 0.3244 for *L.am* and *L.am*-IS, respectively ($p > 0.0013$) as illustrated for two *Leishmania* receptors involved in parasite uptake, the C-type lectin CD209 and the Fcγ receptor 1 (55, 56) (**Figure 4A1**). In contrast, transcripts coding for the costimulatory molecule *cd80* and the immune modulator *cd83* (57) were exclusively increased in response to Ab-opsonized parasites, confirming initiation of the DCs maturation process in accordance to data shown in **Figure 1**.

Amastigote infection increased the abundance of host cell transcripts for molecules involved in classical MHC Class I-restricted antigen presentation, including MHC Class I alpha chain molecules (H2-Q4, Q6, Q7, and H2-K1), and LY75 known to favor antigen cross-presentation (58) (**Figures 4A1, 2**). Transcripts for CD8α, a marker expressed on cross-presenting DC subsets (59) was also increased. In contrast, reduced transcript abundance was observed for MHC class II alpha chains and H2-M molecules (H2-DMB2 and H2-DMA, **Figure 4A3**) known to load peptides onto conventional class II molecules (60).

The changes observed in the cytokine/chemokine transcript profile further supported the stalled DC maturation phenotype observed in **Figure 1**: Increased abundance of *il12b* transcripts correlated with the slight increased secretion of IL12p40 (**Figures 4B1**, **1C2**). In contrast, transcripts for other cytokines known to be secreted during DC maturation (*tnf, il6*) (61, 62) and after inflammasome activation (*il1β, il18*) were down modulated during infection, in accordance to the absence of secretion of these cytokines.

Modulation of some chemokine transcripts, such as *ccl12* or *ccl22* was observed (**Figure 4B2**), which did not translate

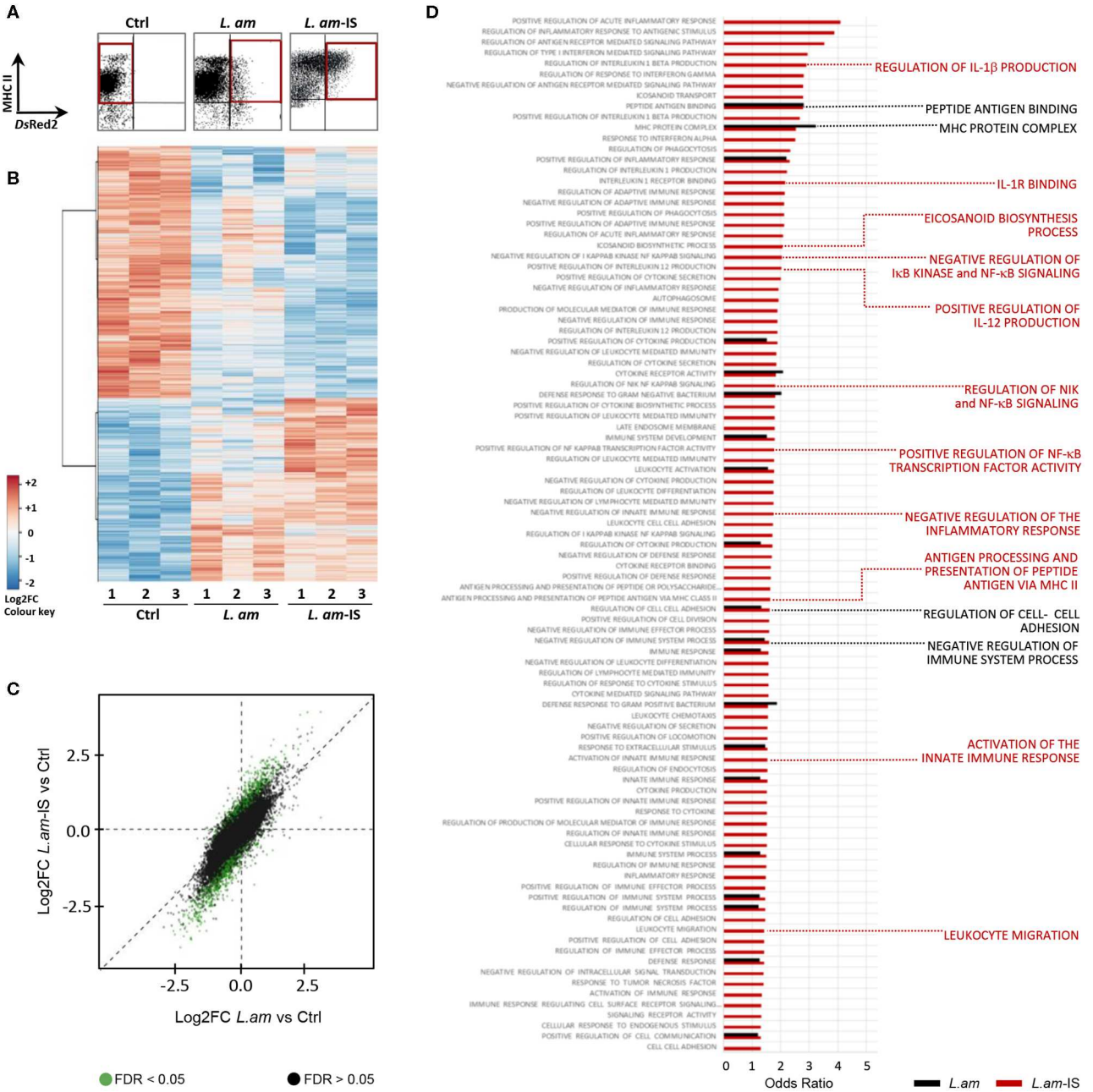

FIGURE 3 | Gene expression analysis in infected DCs enriched by high speed cell sorting. BMDCs alone (Ctrl) or incubated with BCG or *Ds*Red2-transgenic *L. amazonensis* amastigotes without (*L. am*) or with Ab-opsonized (*L. am*-IS) were analyzed. After 24 h, live cells were carefully detached, stained with anti-MHC Class II mAb and sorted with a FACSAria under a BSL-2 cabinet. Sorted DCs were lyzed and total RNA was extracted for transcriptomic analysis. Three independent biological experiments were performed. **(A)** DC isolation by Fluorescence Activated Cell Sorting. The region of interest (red gates) for the sorting of non-infected and infected DCs was defined by the expression of MHC II as determined in uninfected DCs (control, Ctrl), and the presence of the *Ds*Red2 signal of intracellular parasites. **(B)** Global overview of gene expression in sorted samples. Heatmap visualizing differentially expressed Affymetrix probe sets (5% threshold) for sorted triplicate samples (indicated by the number). The color code corresponds to the values of the row-centered expression matrix. **(C)** Illustration of the expression changes between control, *L.am* and *L.am*-IS infected DCs. Log2FC of *L.am*-IS vs. control are plotted (Y-axis) against the log2FC of *L.am* vs. control (X-axis), and dots are displayed in green for probesets differentially expressed (adjusted $p < 0.05$) between *L.am*-IS and *L.am*. The dispersion of green dots indicates a more pronounced transcriptional modulation in DCs infected by Ab-opsonized amastigotes. **(D)** GO enrichment analysis related to DC immune processes in response to *Leishmania* infection. Odds Ratios for key DC biological processes enriched in *L.am* and *L.am*-IS infected DCs compared to control are displayed as black and red bars, respectively. Key DC processes are indicated by the red and black labels.

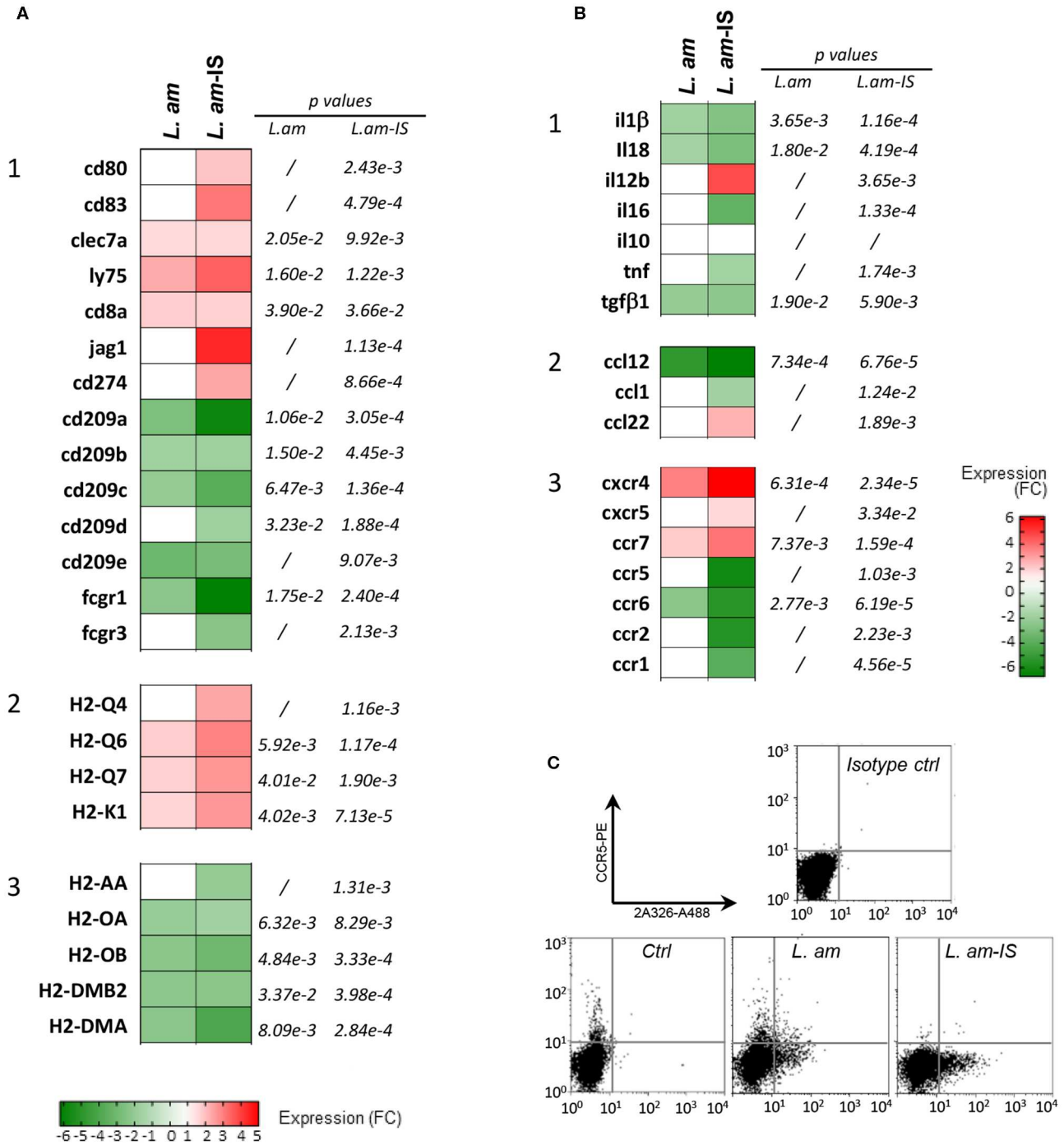

FIGURE 4 | Expression analysis of genes related to the maturation process in *Leishmania*-infected DCs. **(A,B)** Affymetrix analyses were performed on sorted DCs infected with non-opsonized (*L. am*) or Ab-opsonized (*L. am*-IS) *Ds*Red2-transgenic amastigotes. Transcriptional modulation of genes involved in the DC maturation process are displayed as the mean fold change values calculated using uninfected sorted DCs as a calibrator. **(A)** Expression of genes related to maturation and antigen presentation. Heatmaps representing the expression modulation of genes coding for costimulatory molecules and surface receptors (1), MHC I (2), and MHC II (3) molecules. **(B)** Expression of chemokine and cytokine genes. Heatmaps representing the modulation of genes coding for cytokines (1), chemokines (2), and chemokine receptors (3). **(C)** FACS analysis of CCR-5 expression levels. DCs from unsorted cultures were analyzed by FACS for the expression of CCR5, a marker that is rapidly lost during the maturation process. Infected cells were detected using the amastigote-specific, Alexa Fluor-488 conjugated mAb 2A3-26.

into a corresponding increase in secreted proteins after only 24 h of infection (**Supplementary Figure 3**). A number of transcripts for chemokine receptors responded to *Leishmania* infection (**Figure 4B3**), including receptors that are crucial for DC migration: (i) increased expression was observed for *ccr7, cxcr4*, and *ccr2*, with the latter one previously linked to differentiation of protective DCs during *L. braziliensis* infection (63), and (ii) decreased expression was observed for *ccr5* and *ccr6,* which was confirmed at the protein level for CCR5 by FACS analysis (**Figure 4C**). Amastigotes (notably upon Ab-opsonization) thus seem to promote DC motility, which could favor parasite dissemination and visceralization (64).

L. amazonensis Establishes an Anti-inflammatory Phenotype in Infected BMDCs

Transcript profiling revealed that *L. am* infection triggered an anti-inflammatory expression pattern in DCs irrespective of their opsonization status. First, many transcripts related to eicosanoid production were down modulated (**Figure 5A**), including those leading to the synthesis of the leukotriene LTB4, known to amplify NF-κB-mediated responses in macrophages (65) and to be involved in the control of *L. am* infection (66). The increase in *ptges* (Prostaglandin E Synthase) indicates a potential increase in PGE2 levels, which is involved in the suppression of the inflammatory response during human *Leishmania* infection (67). Second, an anti-inflammatory pattern is further supported by (i) down modulation of cytokine receptors involved in signaling cascades leading to NF-κB activation (IL-1 and IL-18 receptor associated proteins and IL-1 receptors), and (ii) up modulation of the IL-1 receptor antagonist (*il1rn*), known to bind to IL-1 receptors and prevent downstream signaling (68) (**Figure 5B**). However, no quantitative difference could be demonstrated for secreted IL1RA as assessed by Cytokine Array (**Supplementary Figure 3**). Third, RNA abundance for the immune-regulatory molecule CD200-known to dampen host microbicidal responses (69) - was increased in DCs infected by Ab-opsonized amastigotes (linear FC = 4.02; p = 8.80E-4). Finally, our data revealed for the first time a coordinated transcriptomic subversion of the inflammasome in DCs infected with non-opsonized (**Figure 5C1**) or Ab-opsonized parasites (**Figure 5C2**), as judged by down modulation of transcripts of various inflammasome components and related cytokines (*caspase-1*, *nlrp3*, *il1β*, *il18*, quadrants 1 in panels C1 and C2), of NLRP3/ASC activators (quadrant 2), and of various complement components involved in inflammasome activation and IL-1β secretion (70, 71) (quadrant 4). In contrast, increased transcript abundance was observed for the NLRP3 inhibitor *tnfaip3* (panel C2, quadrant 3) and *c1qbp,* an inhibitor of the classical complement pathway (panel C2, quadrant 4), thus further reinforcing the anti-inflammatory phenotype. In agreement with the absence of IL-1β secretion (**Figure 2**), this profile indicates that *L. am* infection suppresses NLRP3 priming and activation at four different levels, reminiscent to our recent observation in primary macrophages *in vitro* and *in vivo* (30).

We next assessed the transcriptional effect of infection on the TLR-NF-κB signaling pathway given its essential role of NLRP3 priming (72, 73).

L. amazonensis Amastigotes Subvert NF-κB Mediated Signaling at The Transcript Level

We investigated the impact of infection and parasite opsonization on the TLR/NLRP3-activated, canonical NF-κB pathway, and on the CD40/TNFRSF1b-activated, alternative NF-κB pathway linked to cross-presentation (74, 75). A pleiotropic subversion of the canonical NF-κB pathway was observed following infection with both Ab-opsonized and non-opsonized amastigotes (**Figure 6**, **Supplementary Figure 5**, respectively). Similarly to observations in *Leishmania*-infected macrophages (30), the TLR-NF-κB-NLRP3 axis was inhibited in a dual fashion: (i) by downregulating genes encoding for activators (IL-1 receptors, TIRAP, MYD88, TIFA, EIF2AK2, USP7) and (ii) upregulating genes encoding for negative regulators of the NF-κB pathway (OPTN, TNFAIP3, TAX1BP1, PTPN1, USP10) (**Figure 6**), which was validated at protein level for OPTN (**Figure 2B**), a key inhibitor of NF-κB-mediated immune signaling (76), but not for MYD88 (**Figure 2B**).

In contrast, the observed transcript profile indicates activation of the alternative NF-κB pathway at all levels of the signaling cascade, from the TNFRsf1b surface receptor to the NF-κB2 and RelB nuclear factors (**Figure 6**). The corresponding transcripts were significantly up modulated in response to infection, but no quantitative changes could be evidenced by WB for RELB (**Figure 2B**), whereas the main negative regulator of this pathway *nlrp12* (77) was not affected. The activation of the alternative NF-κB pathway likely causes increased transcription of OPTN, TNFAIP3, and SIRT2, which all are known to further counter-act the activity of the classical NF-κB pathway.

Involvement of Transcription Factor Regulation in Dendritic Cell Subversion by *Leishmania*

The important changes in DC gene expression during *L. am* infection primed us to investigate the potential underlying mechanism of this profound transcriptomic reprogramming. We therefore mined our data sets for changes in the expression profile of Transcription-Related Factors (TRFs), including Transcription Factors (TFs), transcriptional co-regulators and epigenetic factors. Among 2,243 TRFs analyzed (**Supplementary Table 1**), 11.3 and 15.5% showed significant changes in transcript abundance in DCs infected with non-opsonized and Ab-opsonized amastigotes, respectively. These changes correlated with the expression levels of their target genes as revealed by network analysis (**Figure 7**, **Supplementary Figure 6**). For example, decreased expression of the TNF gene correlates with decreased expression of the NFATC1 and NFATC2 transcription factors that regulate key DC immune functions (**Figures 7A1–5**) (78). Likewise, coordinated down-modulation of the MHC Cl II genes H2-Aa, H2-Oa,

A

Symbol	Name	*L.am* FC	*L.am* p value	*L.am* - IS FC	*L.am* - IS p value
Cysltr1	Cysteinyl leukotriene receptor 1	**-2.43**	*8.15E-03*	**-5.64**	*1.49E-4*
Alox5	Arachidonate 5-lipoxygenase	**-2.06**	*5.12E-03*	**-4.73**	*6.46E-5*
Alox5ap	Alox5 activating protein	/	/	**-3.01**	*7.97E-4*
Alox15	Arachidonate 15-lipoxygenase	**-2.33**	*4.01E-02*	**-5.69**	*1.04E-3*
Lta4h	Leukotriene A4 hydrolase	**-1.94**	*1.08E-02*	**-2.73**	*6.70E-4*
Ptgs1	Prostaglandin-endoperoxide synthase 1	**-1.51**	*3.81E-02*	**-2.89**	*3.59E-4*
Ptges	Prostaglandin E synthase	**1.61**	*1.64E-02*	**1.45**	*2.40E-02*
Ptger2	Prostaglandin E receptor 2 (subtype EP2)	/	/	**-2.60**	*3.99E-3*
Ptger3	Prostaglandin E receptor 3 (subtype EP3)	**-3.12**	*9.20E-3*	**-12.29**	*1.09E-4*
Ptger4	Prostaglandin E receptor 4 (subtype EP4)	**1.66**	*1.90E-02*	**2.42**	*7.25E-4*
Hpgds	Hematopoietic prostaglandin D synthase	**-1.63**	*3.97E-02*	**-3.14**	*5.76E-4*
Tbxas1	Thromboxane A synthase 1	/	/	**-2.80**	*7.03E-3*

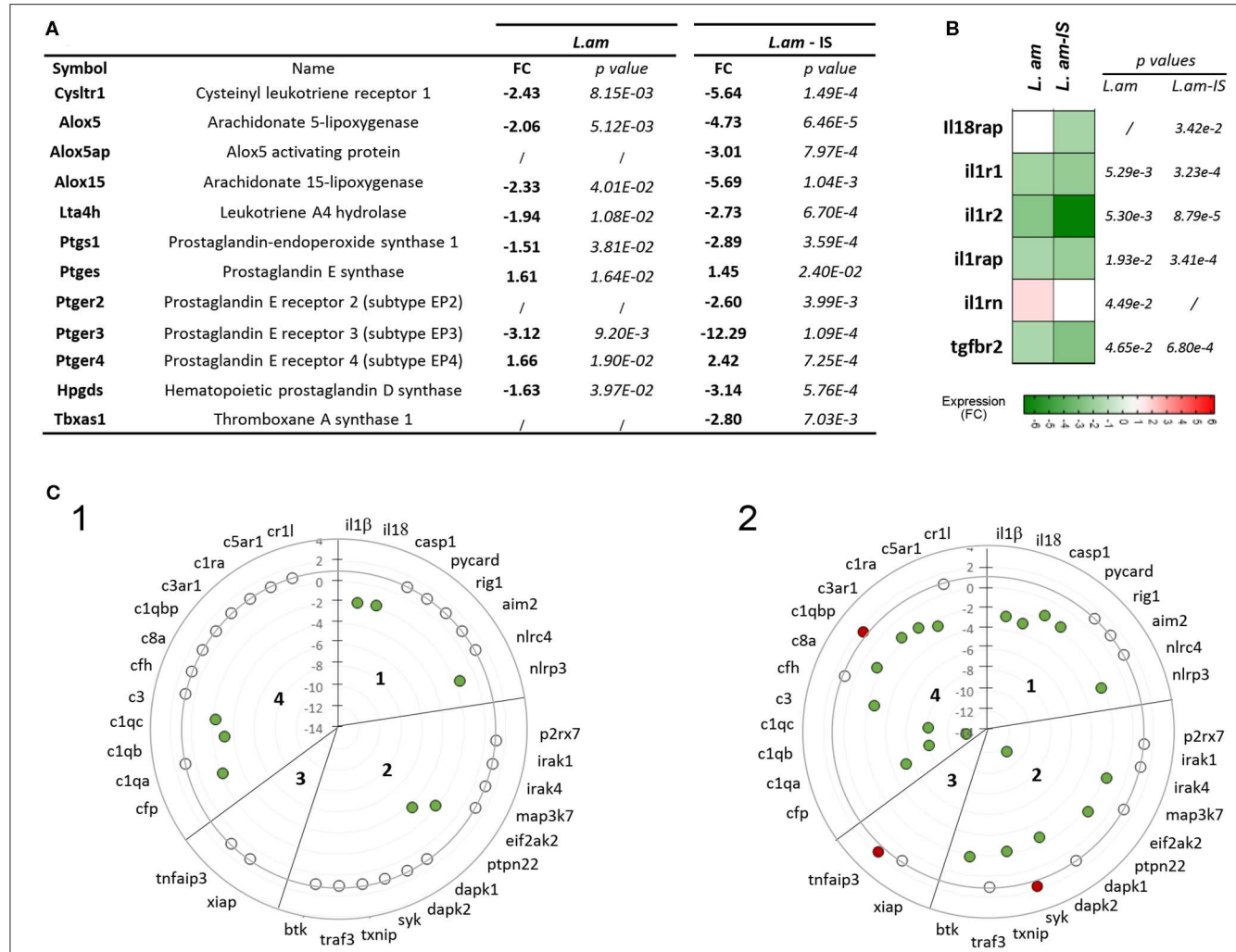

FIGURE 5 | Induction of an anti-inflammatory expression profile in *L.* am-infected BMDCs. Transcriptional modulation observed in the microarray analysis are displayed as mean fold change values calculated using uninfected sorted DCs as calibrator. **(A)** List of differentially expressed genes associated to eicosanoid production. Linear fold changes and *p*-values are indicated for *L. am* and *L. am*-IS sorted DCs. **(B)** Heatmap of transcriptional modulations observed for cytokine receptor genes. **(C)** Transcriptional profiling of genes related to NLRP3 inflammasome priming and activation pathways. Radar plots displaying the modulation of gene expression in sorted DCs infected with non-opsonized (*L. am,* panel 1) and Ab-opsonized (*L. am*-IS, panel 2) *Ds*Red2-transgenic *L. am* amastigotes. The sub-sections correspond to inflammasome-related cytokines and inflammasome components (section 1), NLRP3/ASC activators (section 2), NLRP3 inhibitors (section 3), and components of the complement system (section 4). Unmodulated transcripts are indicated by open dots, whereas up-modulated and down-modulated transcripts are indicated by red and green dots, respectively.

H2-Ob, H2-DMb2, and H2-DMa (**Figure 7A**) correlates with reduced expression of (i) the Class II Major Histocompatibility Complex Transactivator (CIITA) essential for transcriptional activity of the MHC class II promoter (79), (ii) the Forkhead transcription factor FOXO3a that is a key component of the MHC II enhanceosome (80), and (iii) regulatory factor X-associated protein (RFXAP) that binds to the X-box of MHC II promoters (79). These simple regulatory relationships are likely part of more complex regulatory cascades and networks, as suggested by the down-regulation of CIITA itself that could result from the reduced expression of the ETS-domain transcription factor SPI-1 (81) (**Figure 7A**). Vice versa, TF upregulation was correlated with increased target gene expression, as exemplified by the regulatory relationship between NLRC5 and MHC Class I molecules (82, 83), REL and IL12b (84), RELB and CD80 and MHC Class I molecules (85) or IRF8 and LY75 (86) (**Figure 7B**).

Our network analysis further reveals that reduced IL-1β expression is likely the result of a dichotomic regulatory event, with *L. am* infection reducing the expression of its direct transcription factors (SPI-1 and HIF-1α) (87, 88), while at the same time increasing the expression of the TFs RELB and RORA that have a negative effect on IL-1β expression (89–91) (**Figure 7C**). Unlike previously observed in *L. am*-infected macrophages (30), the expression of the pro-inflammatory NF-κB family member RELA is not modulated in BMDCs. However, the reduced expression of (i) upstream actors of the classical NF-κB pathway, including TLR2 and TLR3 that are also expressed

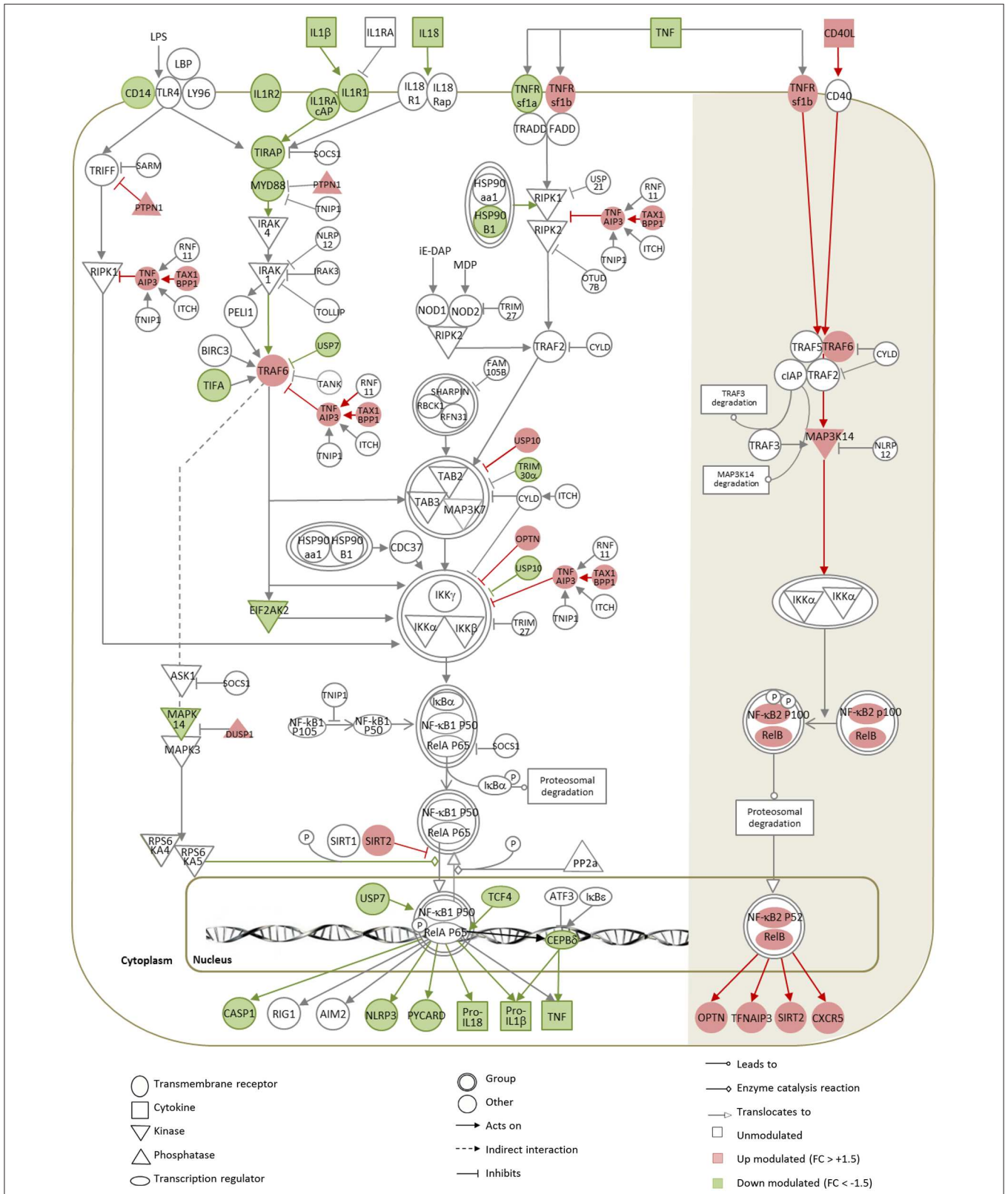

FIGURE 6 | Gene expression map of the NF-κB pathway in DCs infected with Ab-opsonized amastigotes. Significant modulations calculated between *L.am*-IS-infected and uninfected BMDCs are represented by the color code, with red indicating up-regulated (linear FC > +1.5) and green down-regulated (linear FC <-1.5) genes. Symbols, lines, and color codes are defined in the legend. White (left) and shaded (right) areas correspond to the classical and to the alternative NF-κB pathways, respectively.

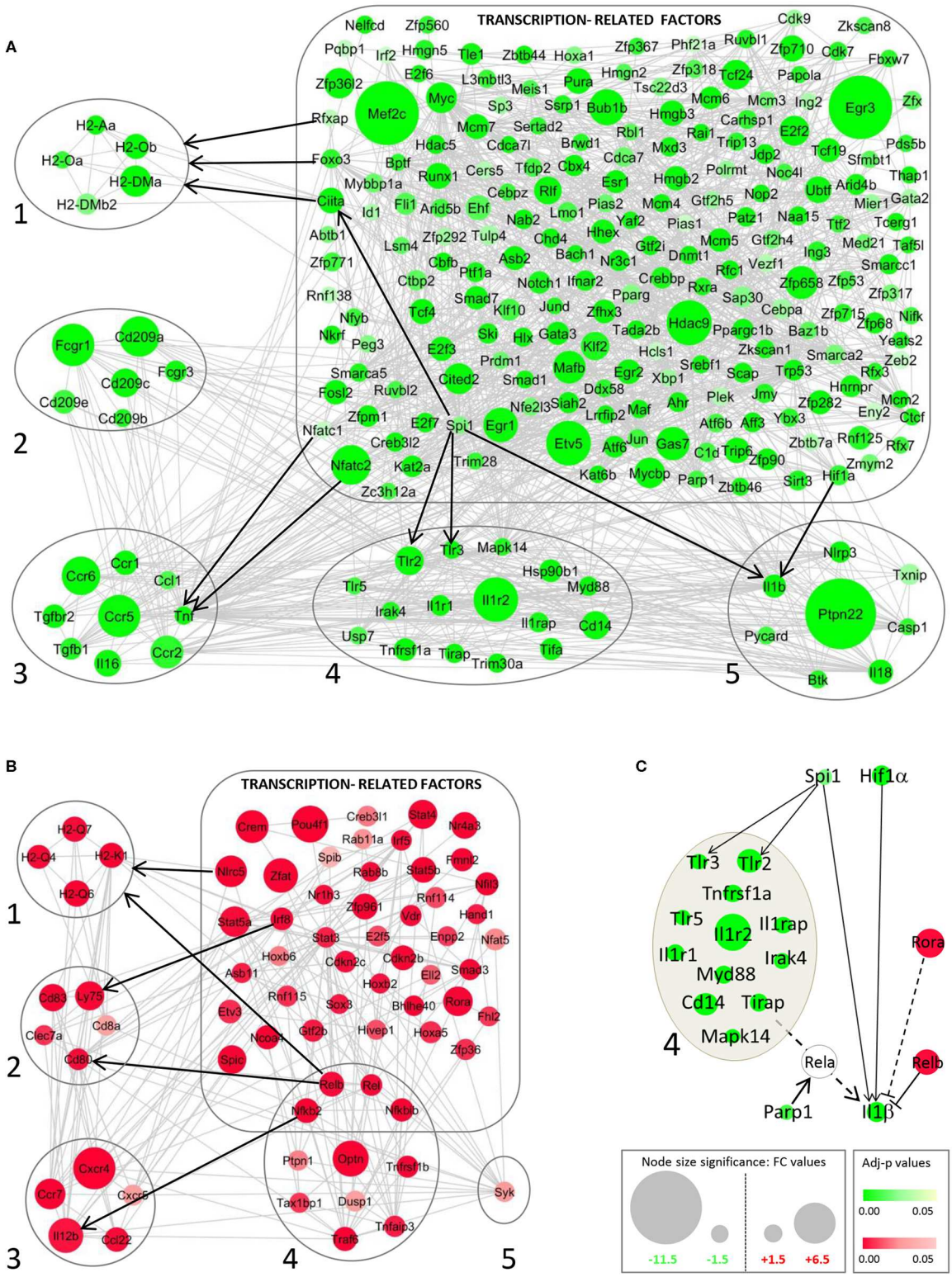

FIGURE 7 | STRING network analysis for modulated genes of transcription-related factors (TRFs) in DCs infected with Ab-opsonized amastigotes. Networks for down-modulated (green) **(A)** and up-modulated genes (red) **(B)** are shown. Interactions between TRFs and MHC II (1), DC markers/receptors (2), cytokine/chemokine receptors (3), TLR/Cytokine receptors and the NF-κB pathway (4), and inflammasome-related molecules (5) are depicted. Gray lines correspond to active interactions (settings used for STRING analysis) and black arrows exemplify key TRF – target gene interactions validated by publication. **(C)** Integrative network of modulated TRFs and their related target genes involved in regulation of the IL-1β gene. Dotted lines correspond to indirect interactions (mediated by RelA, related to the TLR/NF-κB axis, group 4) on the IL-1β gene.

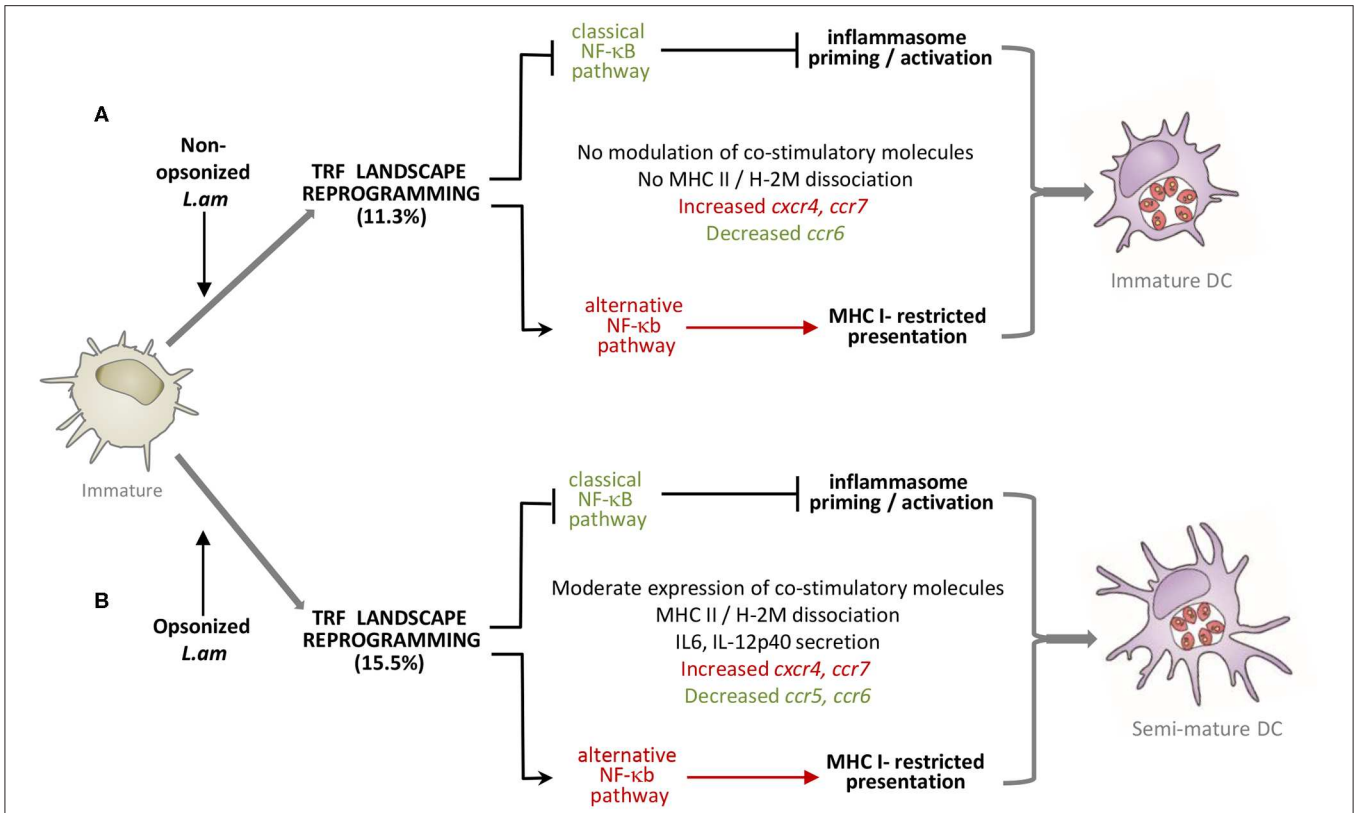

FIGURE 8 | Model of DC subversion by *L. am* amastigotes. Transcriptomic and phenotypic modulations induced by non-opsonized **(A)** and Ab-opsonized **(B)** *L. am* amastigotes in immature BMDCs are shown. In both cases, inhibition of the classical NF-κB pathway is linked to prevention of inflammasome priming and activation. The alternative NF-κB pathway is favored in both infection conditions, likely promoting MHC I-restricted antigen presentation.

under the control of SPI-1 (92, 93), and (ii) the Poly(ADP-ribose) polymerase-1 (PARP1) gene, which encodes for a nuclear chromatin-associated protein known to co-activate NF-κB-dependent transcription (94), likely prevents RELA-dependent IL1β gene expression (**Figure 7C**).

Together, these data reveal that the subversion of the DC transcriptomic landscape during *Leishmania* infection may be the consequence of subversion of key TFs that regulate the host cell immune response.

DISCUSSION

Using a FACS-based sorting procedure and applying systems analyses on transcriptomics data, we uncovered a novel mechanism of DC immune-subversion by non-opsonized and Ab-opsonized, virulent *Leishmania amazonensis* amastigotes. This subversion targets transcription-related factors (TRFs), which in turn causes important changes in expression of immune-related genes, interferes with DC maturation (**Figure 8**) and favors persistent parasite infection.

Leishmania parasites are capable to alter key immune functions of DCs, including maturation and migration properties (8–11). The subversion of these essential functions are mediated by the inhibition of various signaling pathways, including pro-inflammatory transcription factors of the STAT and NF-κB protein families (10, 11, 13, 14, 95, 96). Our data largely extend this list to many other TFs, i.e., *spi1*, *tcf4*, *myc*, *zbtb46*, *irf2*, *irf8*, *relb*, *pparg*, *spib*, or *nfil3* (97) that show parasite-driven inhibition of expression in infected DCs, raising important questions on the underlying mechanisms that allow such coordinated modulation of the TRF landscape. In macrophages, *Leishmania* infection has profound effects on the macrophage epigenetic profile, both at the level of DNA methylation (98) and H3 histone post-translational modifications (30, 99, 100). We recently provided first evidence that *L. am* amastigotes induce histone H3K9/14 hypo-acetylation and H3K4 hypo-trimethylation at promoters of NF-κB-related, pro-inflammatory genes in infected macrophages *in vitro* and in infected tissues *in vivo* (30). Based on these results, it is interesting to speculate that the expression changes in TRFs are caused by changes in the activity of histone modifying enzymes (HMEs) or DNA methyltransferases (DNMTs). Indeed, epigenetic regulation controls DC development, immune functions and phenotypic heterogeneity (101), and *L. am* may have evolved strategies to exploit this remarkable plasticity of DCs by interfering with the host cell's epigenetic profile. Our data provide first insight into a possible, reciprocal regulatory relationship between TRFs and

HME/DNMT activities that may govern DC reprogramming: Mining our data sets we observed reduced expression in infected DCs of *dnmt1,* the histone deacetylases *hdac5* and *hdac9, sirt3*, and various TFs known to remodel the epigenetic landscape such as *cebpa* or *nfatc2* (102, 103).

The interplay between transcriptional and epigenetic regulation in establishing an anti-inflammatory phenotype in *L. am*-infected DCs is further illustrated by the factors controlling IL-1β expression, notably the NF-κB family members *rela* and *relb*. While the activation of the canonical NF-κB pathway depends on the rapid and transient nuclear translocation of RelA/p50 dimers, the non-canonical pathway is activated in a slow and persistent manner via a RelB/p52 complex (77, 104). This second pathway plays a critical role in regulating immune homeostasis, and its dysregulation contributes to inflammatory and autoimmune diseases (77, 105–107) suggesting that RelB may act as a repressor of NF-κB-responsive gene expression. Indeed, our data link increased expression of *relb* with inhibition of IL-1β expression during *Leishmania* infection, likely by changing the chromatin structure at the IL-1β promoter causing epigenetic silencing (89, 90).

Subversions of the TRF landscape in *L. am*-infected DCs had a profound effect on the host cell immune status, in particular on the TLR/NF-κB immune axis - a key signaling pathway regulating DC functions (16, 108, 109). This pathway was inhibited both at the transcriptional and signaling levels, resulting in reduced expression and secretion of chemokines and pro-inflammatory cytokines, absence of inflammasome activation, and stalling of the maturation process in *L. am*-infected DCs (**Figure 8**). Increased expression of TNFAIP3 and OPTN, two negative regulators of the TLR/NF-κB/NLRP3 axis, indicates that this pathway may be even suppressed in infected DCs as previously demonstrated in *L. am*-infected macrophages (30). Such a suppression is further sustained by the down-modulation of positive regulators of the TLR-classical NF-κB axis (e.g., IL1 receptors, TNFRsf1a, TIRAP, MYD88, TIFA, USP7) and increased expression of members of the alternative NF-κB pathway (e.g., TNFRsf1b, TRAF6, MAP3K14, NF-κB2, RelB), a similar regulatory dichotomy we recently uncovered in *L. am*-infected macrophages (30). Surprisingly, the anti-inflammatory state observed during DC infection was further enhanced by parasite Ab-opsonization, a *Leishmania*-specific signature that contrasts with the efficient DC maturation and inflammasome activation observed with other intracellular pathogens, including *Cryptococcus neoformans*, *Staphylococcus aureus, Escherichia coli* or *Francisella tularensis,* whose opsonization triggers IL-1β secretion (110–112). In contrast, MHC Class I-restricted presentation and cross-presentation seems to be promoted in *L. am*-infected DCs, confirming previous reports (113, 114). Based on our data, this response may be favored by increased expression of the mannose receptor LY75 (58), members of the alternative NF-κB pathway (115, 116), MHC Class I molecules, NFIL3 (117), the small GTPase RAB11A involved in receptor signaling (118), and the decreased expression of the m^6A-marked mRNA binding molecule YTHDF1 (119, 120).

In conclusion, we describe a new *Leishmania* immune subversion strategy resulting in stalled DC maturation and pleiotropic inhibition of the TLR/NF-κB/NLRP3 axis, which may have important phenotypic and immunologic consequences: Preventing IL-1β secretion may hamper expansion, survival, and migration of antigen-primed $CD4^+$ and $CD8^+$ T cells, and Th1, Th2, and Th17 differentiation (18, 20, 121). Indeed, we observed increased transcript expression of *cd8a*, *ly75* (DEC205), *jag1,* and *cd274* in response to *L. am* infection, suggesting the induction of tolerogenic DCs (122, 123), which may favor the differentiation of anergic or regulatory T cells (124, 125), thus causing immune suppression and favoring *Leishmania* infection and immunopathology (126). Our results will incite future studies aimed to characterize the transcriptional landscape and antigen presenting capacity of DCs *in vivo* that can likely be modulated directly by intracellular *Leishmania*, indirectly by the uptake of parasite remnants, or by the local immune response, notably pro- (e.g., TNF) and anti- (e.g., IL-10) inflammatory factors produced by bystander cells. Our study will open interesting new avenues for the design of anti-parasitic immuno-therapies targeting DC epigenetic and transcriptional control to rescue the DC's key functions in mounting an efficient, anti-leishmanial T cell response.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO: GSE144039; secure token= khahiiccdjgjxyx.

ETHICS STATEMENT

All animals were housed in A3 animal facilities according to the guidelines of Institut Pasteur and the "Comité d'Ethique pour l'Expérimentation Animale" (CEEA) and protocols were approved by the "Ministère de l'Enseignement Supérieur; Direction Générale pour la Recherche et l'Innovation" under number 2013-0047 and by the Animal Care and Use Committee at Institut Pasteur of Shanghai Animal Care.

AUTHOR CONTRIBUTIONS

HL, EP, GMi, GS, and GMe: study design. TR, HL, and P-HC: acquisition of data. HL, EP, TR, KK, and AL: analysis and interpretation of data. HV, EP, and HL: statistical analyses. HV and EP: organized the database. GMi and RW: material support. HL, EP, TR, KK, P-HC, and J-YC: technical support. HL, EP, GMi, GMe, and GS: drafting of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01098/full#supplementary-material

Supplementary Table 1 | List of transcription factors and co-regulators. Gene symbols are listed with their Ensembl reference and short description.

Supplementary Table 2 | Partial list of GO gene sets highlighted in *Leishmania*-infected DCs. Gene-level results corresponding to 18 selected GO terms significantly ($p < 0.05$) evidenced in DCs infected with *Leishmania amazonensis* amastigotes (*L. am* and *L. am*-IS) are presented. The summary sheet describes the GO terms and their corresponding sheet (S2 to S19). For each GO term, genes are listed with their Entrez number, probe-set ID, Fold Changes (Log base 2) and adjusted *p* values for both *L. am* vs Ctrl and *L. am*-IS vs Ctrl comparisons. Color code indicates *p* values below the 5% threshold level.

Supplementary Figure 1 | Evaluation of the maturation status of DC cultures by epifluorescence microscopy. BALB/c-derived BMDCs alone (Ctrl) or incubated for 24 h with BCG (BCG) or *L. amazonensis* amastigotes without (*L. am*) or with Ab-opsonized (*L. am*-IS) were subjected to epifluorescence microscopy imaging of MHC Class II and H2-M molecule staining. Representative DCs from the different conditions are displayed as merged images of the 3 detection channels showing MHC II molecules (green), H2-M molecules (red), and nuclear DNA (blue).

Supplementary Figure 2 | Influence of the γ chain subunit of FcgammaRI, FcgammaRIII, and Fcepsilon RI receptors on the phenotype of DC cultures. **(A)** Flow cytometric comparison of surface marker expression of BMDCs from wild type mice from Charles River (CR) and Taconic (Tac) laboratories. Histograms show the mean fluorescence intensity of the indicated markers. Numbers represent the percentage of DCs expressing the corresponding marker. Note the similarity of the profiles between both sources of BALB/c mice. **(B)** Phenotypic analysis of DCs derived from wild type (gray histograms) and $Fc\gamma^{-/-}$ (black histograms) mice from Taconic laboratories. DCs alone (Ctrl) or incubated for 24 h with BCG or *L. amazonensis* amastigotes without (*L. am*) or with Ab-opsonization (*L. am*-IS) are shown. Histograms correspond to the mean fluorescence intensity of the indicated markers. Numbers represent the percentage of DCs expressing the corresponding marker. Note that the absence of the γ chain abrogates the marker increase observed in *L. am*-IS-infected DCs from WT background.

Supplementary Figure 3 | Results of cytokine array analysis of DC culture supernatants. Uninfected BMDCs (ctrl, **A**), or BMDCs infected with non-opsonized *L. am* amastigotes **(B)**, Ab-opsonized *L. am* amastigotes **(C)** or live BCG **(D)** are shown. Supernatants were analyzed using the mouse XL cytokine array kit. Pictures of the membranes are shown after revelation and scanning. Cytokines and chemokines already expressed in control cultures are labeled in black. Those specifically detected in the BCG-treated culture are labeled in red.

Supplementary Figure 4 | Quality control of RNAs and microarrays for sorted DCs. Uninfected BMDCs (ctrl), or BMDCs infected with non-opsonized *L. am* amastigotes (*L. am*), Ab-opsonized *L. am* amastigotes (*L. am*-IS) were detached, stained with PE-CY5-conjugated anti-MHC II mAb and sorted without fixation by a high-speed sorting procedure. The sorting was performed with a FACSAria in a BSL-2 containment ($n = 3$ independent experiments). **(A)** Evaluation of RNA integrity after electrophoresis using Agilent Lab-on-chips. Total RNA extracted from sorted infected DCs and control uninfected DCs were analyzed. RNA integrity numbers (RIN) are indicated for representative samples. **(B)** Modulation of DC gene expression by *Leishmania* amastigotes. Differentially expressed probe-sets (adjusted $p < 0.05$) between sorted non-opsonized *L. am*-infected DCs and control cells are represented in red. Ab-opsonized amastigotes (*L.am*-IS) induce a much stronger effect than non-opsonized ones (*L.am*).

Supplementary Figure 5 | Gene expression map of the NF-κB pathway in DCs infected with non-opsonized amastigotes of *L. amazonensis*. Significant modulations calculated between *L.am*-infected and uninfected BMDCs are represented by the color code, with red indicating up-regulated (linear FC > +1.5) and green down-regulated (linear FC <-1.5) genes. Symbols, lines and color codes are defined in the legend. White (left) and shaded (right) areas correspond to classical and alternative NF-κB pathways, respectively.

Supplementary Figure 6 | STRING network analysis for modulated genes of transcription-related factors (TRFs) in DCs infected with non-opsonized amastigotes. Networks for down-modulated (green) **(A)** and up-modulated genes (red) **(B)** are shown.

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Behind Enemy Lines: Immunomodulatory Armamentarium of the Schistosome Parasite

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The deeply rooted, intricate relationship between the *Schistosoma* parasite and the human host has enabled the parasite to successfully survive within the host and surreptitiously evade the host's immune attacks. The parasite has developed a variety of strategies in its immunomodulatory armamentarium to promote infection without getting harmed or killed in the battlefield of immune responses. These include the production of immunomodulatory molecules, alteration of membranes, and the promotion of granuloma formation. Schistosomiasis thus serves as a paradigm for understanding the Th2 immune responses seen in various helminthiases. This review therefore aims to summarize the immunomodulatory mechanisms of the schistosome parasites to survive inside the host. Understanding these immunomodulatory strategies not only provides information on parasite-host interactions, but also forms the basis in the development of novel drugs and vaccines against the schistosome infection, as well as various types of autoimmune and inflammatory conditions.

Keywords: schistosomiasis, immunomodulation, innate immunity, parasite, Th2 immune response, cytokines

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INTRODUCTION

Immunomodulation is a tactic employed by parasites to successfully invade their human hosts. As an adaptive survival skill, helminths employ a great diversity of immunomodulatory strategies for evading immune detection, suppressing cellular immunity, and eluding host immune attacks (1, 2). These promote their longevity inside the host to further continue their life cycle and facilitate transmission.

Unlike the rapidly multiplying protozoan parasites, some of which use antigenic variation as an effective evasion strategy in escaping immune recognition (3), the chronicity of helminthic infections has led helminths to act against the host immune responses by downmodulating the latter's intensity and effectiveness. *Schistosoma* parasites have been shown to induce the Th2 response that is shown to be more favorable to important biological processes inside the host such as migration and egg excretion (4). During its intra-mammalian life cycle, *Schistosoma* needs to conquer a war zone consisting of the host's innate and adaptive immune responses. The life stages of the parasite that will have to contend with the host's immune system are the penetrating cercariae, the migrating schistosomula, the adult worms, and the eggs produced by the adults *in copula*.

During schistosome infection in the mammalian host, cytokines play major roles in the regulation of immune and inflammatory responses against invading parasites. These effector molecules, particularly those produced by the immune cells, not only mediate both physiological and pathological consequences at the onset of immune response, but also control the degree and duration of such a response. The schistosome parasites are therefore equipped with

immunomodulatory armamentarium acting as counter-defenses to protect themselves from the destruction brought about by host immune attacks. This review aims to provide a summary of these immunomodulatory strategies that might be crucial for the survival of the schistosomes within their definitive host.

INFILTRATING HOST TERRITORY

The skin is the largest organ of the body and consists of a complex network of different cell types that maintain several vital processes including immune responses for disease prevention. The schistosome cercariae begin their invasion by infiltrating the skin- the host's primary defense. During invasion, the parasite needs to ensure its survival by orchestrating immune regulation within the skin. The percutaneous entry of the schistosomula elicits an inflammatory response characterized by infiltration of polymorphonuclear cells (PMNs) and mononuclear cells (5, 6). Localized production of pro-inflammatory cytokines including interleukin (IL)-1b, IL-6, IL-12, and tumor necrosis factor (TNF)-α (7, 8) is supposed to promote this pro-inflammatory Th1 response. However, invasion by the schistosome paradoxically leads to predominantly Th2 immunity. This skewing of the immune response arises from the production of certain immunoregulatory mediators. IL-10, produced by keratinocytes, macrophages, dendritic cells (DCs) and B1 lymphocytes, is one of these key immunomodulatory cytokines elaborated in the skin in response to the cercarial invasion (9). In addition, a major molecule from the secreted protein of the cercaria known as Sm16 has been shown to modulate innate immunity by preventing macrophage classical activation and delaying antigen processing (10). Sm16 is also capable of blocking the activation of IL-1 receptor-associated kinase 1 (IRAK-1) gene, which is important in the production of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) (11). NF-κB is known to be a key player in the regulation of immune responses to infection (12).

Excretory-secretory (ES) products from the cercaria likewise stimulate inhibitory molecules like prostaglandins. Studies have shown that schistosomula can induce prostaglandin E2 (PGE2) production in the human keratinocytes (13). This over-expression of PGE2 in the skin plays an important role in the production of IL-10 through a cyclooxygenase 2-dependent pathway (14). In addition, PGE2 is also a potent vasodilator (15) aiding the passage of the schistosome into the circulation. In fact, in a murine model for *Schistosoma mansoni*, PGE2 has been documented to be the main immunoregulatory molecule in the skin (13).

Langerhans cells (LCs) are considered the first-line fighters in the skin considering their location in the outer layers as compared with other types of DCs (16). LCs are known to induce immunological tolerance (17, 18), and their suppressive effects arise from IL-10 production and CD4+ regulatory T cells induction (19). When the skin is invaded by pathogens such as schistosomes, keratinocytes and LCs produce pro-inflammatory cytokines such as TNF-α and IL-1b stimulating the actin-dependent migration of the LCs (20). Another prostaglandin, now produced as a component of the schistosomula's ES proteins, prostaglandin D2 (PGD2) together with PGE2 leads to increased production of IL10 (21). The anti-inflammatory IL-10 downregulates the production of both IL-1b and TNF-α, thus inhibiting the migration of epidermal LCs to the site of invasion (21). Overall, this disruption in the movement of antigen presenting cells (APCs) from the site of exposure to the draining lymphoid tissue is a vital immunomodulatory strategy adopted by the *Schistosoma* parasites (21).

Mast cells (MCs) are another key player in the immune response against parasitic infections. MCs are abundant near cutaneous and mucosal body surfaces where early immune surveillance occurs. The schistosome parasite has been shown to release ES proteins that can induce mast cell degranulation (22). One of these molecules is likely to be the schistosome homolog of the human translationally controlled tumor protein (TCTP) that has been shown to degranulate both basophils and mast cells (23). Binding of histamine from activated mast cells to H2 histamine receptors induces IL-10 production in maturing DCs (24, 25) and inhibits the production of Th1 promoting cytokine IL-12, which in turn is a powerful inducer of interferon- γ (IFN-γ) (26). This results in matured DCs polarizing naive CD4+ T cells toward the Th2 phenotype (24).

Parasites can also regulate the host's immune response by inducing apoptosis of host cells (27). A 23 kDa protein called *S. mansoni* apoptosis factor (SMAF) has been characterized as a component of the cercarial ES products that can trigger apoptosis in the CD4+ lymphocyte population via Fas–FasL interaction. The same study suggests that the CD4+ cell apoptosis modulates the host's immune response and allows the schistosome parasite to evade immune surveillance (28).

Studies have also shown that ES products from the schistosomula stimulate APCs toward Th2 immune responses. ES-activated DCs trigger CD4+ cells to produce regulatory cytokines IL-4, IL-5, and IL-10-, all indicative of a Th2 response (29). Furthermore, these DCs also lose the ability to produce Th1-promoting cytokines including IL-12, IL-23, and IL-27 (30). It thus appears that immunomodulatory molecules in the ES products could modify the APCs to promote Th2 responses over the Th1 phenotype (31).

CAMOUFLAGING OF THE MIGRATING SCHISTOSOMULA

Skin-stage schistosomules are susceptible to both humoral and cellular immune responses. However, the significant morphological and biochemical changes occurring in the developing schistosomula render them resistant to the host immunological defenses (32), as seen in the lung schistosomulum (33, 34). These changes include shedding of the cercarial membrane and formation of the heptalaminate surface membrane (35). This unique outer-surface tegumental membrane might be an adaptation to resist host immune effectors such as complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC) (36, 37). Different immune evasion strategies have been proposed to explain the inefficient

host immune response against the exposed schistosome tegument (37). These include rapid tegument turnover, masking with acquired host antigens, and poor immunogenicity of exposed antigens (38).

Danger-associated molecular patterns (DAMPs) are tissue-derived distress signals released during stress or injury. One such DAMP is extracellular ATP involved in purinergic signaling (39). Extracellular ATP has been shown to induce the degranulation of neutrophils and the production of pro-inflammatory cytokines in macrophages and monocytes (40). Ecto-enzymes, such as alkaline phosphatase, phosphodiesterase, and ATP diphosphohydrolase, have been found to be expressed in the tegument of schistosomula (41–43). These ecto-enzymes might catalyze the conversion of ATP to adenosine and effectively degrade DAMPs released by host cells in response to intravascular schistosome migration, interfere with purinergic signaling, thus preventing pro-inflammatory responses, and subsequently lowering host immunity against the parasite (44).

Aside from their role against the pro-inflammatory ATP, these ecto-enzymes also inhibit blood coagulation in the tissue vicinity (45). Platelets themselves have been shown to damage the schisosome parasite (46). The catabolism of ATP and ADP through the ecto-enzymes characterized in *S. mansoni,* including the tegumental ecto-apyrase ATP diphosphohydrolase (SmATPDase-1) (47), alkaline phosphatase (SmAP) (48), and phosphodiesterase (SmNPP5) (49), may lead to the inhibition of platelet aggregation and thrombus formation around the worm. Moreover, activated platelets and immune cells release inorganic polyphosphates (polyPs) (50, 51). polyPs are essential for the activation of factor XII, which triggers the kallikrein-mediated kininogen pathway, thus producing high levels of bradykinin, increasing vascular permeability, and promoting inflammatory responses (52). SmAP has been shown to hydrolyze polyPs *in vitro* thereby possibly preventing their action against the parasite (48). This SmAP-mediated cleavage of polyPs may therefore contribute to the survival of the intravascular stages of the schistosome parasite, including the schistosomula and the adult pairs within their hostile habitat (48).

Lung schistosomula need to resist immune damage as they have been shown to activate complement (53) and bind antibodies on their surface membrane (54). Therefore, the structural and biochemical modifications of the schistosomulum's surface membrane tend to produce immunological camouflage that either prevent antibody binding or effectively reduce antigen expression (32). Furthermore, caveolin-like molecules and membrane fractions characteristic of detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) or detergent-resistant membranes (DRMs) have been observed on the surface membrane of the schistosome (55), thus indicating the presence of lipid-rafts (56) that might serve as an additional protection for the parasite. Lipid rafts are presumed to enable signal transduction by selectively concentrating intracellular signaling molecules in which protein kinases, scaffolding molecules, and substrates are in close proximity (57). In schistosomes, the lipid rafts have been shown to possess specialized signaling domains such as protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) (58). PKC and ERK are important mediators known to regulate diverse processes in eukaryotes such as growth, development and differentiation, cell cycle, motility, apoptosis, and survival (59, 60).

Moreover, genes associated with immune evasion and stress responses, such as the potent anti-inflammatory Sm-16 and paramyosin, are over-expressed in lung schistosomula (61). Sm-16 might play a crucial role in the interaction of the parasite with immunoreactive lung microvasculature endothelial cells during the passage of the schistosomulum through the lung (62). On the other hand, paramyosin, found on the tegumental surface of the schistosomula, aids in immune evasion through its receptor that is capable of adsorbing antibodies onto the parasite surface at the latter's Fc regions (62, 63).

Nitric oxide (NO) plays a very important role both in the mammalian hosts and in helminths with respiratory pathology (64). It is a key messenger in the pathogenesis of inflammation by acting as a signaling molecule during T cell-mediated immunity (65). IFN-γ up-regulates inducible nitric oxide synthase (iNOS) leading to the production of NO (66). This cytokine is produced by the immune effector CD4+ T cells as an immune response against the schistosomula in the lungs (66). In an experimental study comparing the susceptibilities of different stages of larvae to killing by NO, lung schistosomula obtained 1 week after infection were not killed *in vitro* by NO generated either from a chemical NO donor or from activated cells (67). At this period, the schistosomula has been shown to undergo anaerobic metabolism (68), thus negating the aerobic metabolism-dependent effects of NO against the parasite (67). During transformation of cercaria into schistosomulum, the parasite rapidly shifts from carbon dioxide production via the Krebs cycle to lactate production using glycolysis (69), and from consumption of stored glycogen to dependence on host glucose as fuel (70). Furthermore, schistosomula have higher levels of mRNAs associated with anaerobic glucose metabolism (70) and lower expression of respiratory enzymes (71). As the schistosomes develop into adults, however, they regain a significant capacity to produce energy via aerobic metabolism (70).

Once the schistosomulum becomes successful in evading the host's immune response, it goes into the portal veins and matures into an adult over a period of 1–3 weeks. The male and female adult schistosomes pair up, adhere to the veins, bring forth 300–3,000 eggs, and escape host immunity for many years.

SURVIVAL OF THE ADULT PAIRS IN THE VASCULAR SYSTEM

The major task of the adult schistosomes is to produce eggs while surviving within the vascular system of the host. The circulatory system is home to various immune defenses including immune cells, phagocytes, complement proteins, and antibodies. However, the adult schistosomes are capable of avoiding the immune recognition system by coating their outer tegument with antigens from the hosts. Several studies have shown that the adult *Schistosoma* parasites were covered with immunoglobulins,

β2 microglobulin, complement components, and other host antigens (72–75).

The complement system is an essential component of innate host immunity, and therefore schistosomes should protect themselves from complement-dependent cytotoxicity. To avoid complement-mediated auto-hemolysis, host erythrocytes are provided with a 70-kDa glycosylphosphatidylinositol (GPI)-anchored protein known as decay accelerating factor (DAF), which inhibits C3 convertases in both the classical and alternative pathways of the complement system (76). An *in vitro* experiment showed that adult schistosomes were capable of abstracting DAF or CD55 from host erythrocytes, which then serves as a valuable defense against the action of the complement system (77). The adult schistosomes are also provided with inhibitors of human complement activation on their tegument such as the trispanning orphan receptor of *S. haematobium* (Sh-TOR), a receptor that can bind specifically to human complement C2 (78). Finally, paramyosin is a known inhibitor of the complement membrane attack complex. It has been discovered that earlier known complement inhibitor SCIP-1 (34) is just a surface-exposed form of paramyosin (79). Paramyosin might therefore have some significance in the immunomodulation by inhibiting the activation of the terminal pathway of the complement system (79).

SKEWING OF IMMUNE RESPONSE

Following schistosomule migration, a Th1 immune response is elicited as characterized by a marked increase in IL-1 and IFN-γ induced by the worm antigens (80). The Th1 response persists for approximately 5 weeks. However, as the parasites mature, the immune response is skewed into the Th2 type (30). Experimental single-sex infections in mice models have shown that both male and female worms individually induce IL-4 production by $CD4^+$ T cells and promote a Th2 response even before eggs are laid (31).

Toll-like receptors (TLRs) are a family of pattern recognition receptors expressed in cells of the innate immune system such as macrophages and DCs (81). Activation of TLRs induces Th1 immune response with a predominant production of IFN-γ by the CD4+T cells (82), in addition to Th1 promoting cytokines IL-12, IL23, and IL27 secreted by APCs (83). In *S. mansoni*, the TLR2 and TLR4 of DCs have been shown to recognize the schistosome specific phosphatidyl serine-containing lipid antigen lysophosphatidylserine (lyso-PS) (84), and lacto-N-fucopentose III (LNFPIII), respectively, in the worm's ES (85). These TLR-mediated signaling reduces the ability of the DCs to produce IL-12 and promotes a polarization toward a Th2 immune response instead of the Th1 type. Ligation of LNFPIII and the TLR4 in DCs by *Schistosoma* induces phosphorylation of mitogen-activated MAP kinase (MAPK) ERK (85). On the other hand, the schistosomal lyso-PS has been shown to induce activation of DCs promoting Th2 and regulatory T cell development via a TLR2-dependent mechanism (84). TLR2 ligation stabilizes the MAPK ERK, and stimulates the transcription factor c-Fos, thereby suppressing IL-12 production, and promoting polarization toward Th2 immune responses (86).

At 5–6 weeks post-infection, the adult female schistosomes start to release eggs after pairing with the male worm. The schistosome eggs evoke a host immunity that is more robust compared with the ineffective response mounted against invading cercariae and adult worms (87). Eggs of *S. haematobium* have been shown to elicit an immediate, initial response within 24 h upon release, marked by the induction of pro-inflammatory mediators such as TNF-α, on one end, and that of anti-inflammatory cytokines that include CCL11 (88). Schistosome eggs, viable or dead, are remarkably capable of inducing Th2 responses (89). This Th2 phenotype is characterized by the proliferation of Th2 cells, eosinophils, and basophils; elevated production of immunoregulatory cytokines IL-4, IL-5, and IL-13; and polarization of antibodies toward the IgG1 and IgE isotypes, and of macrophages toward M2 phenotype (90, 91). The ES proteins, such as the dimeric glycoprotein alpha-1 (α1) or IL-4 inducing principle of schistosome eggs (IPSE), and the hepatotoxic egg glycoprotein omega-1 (ω1), thus play important roles in the immunomodulation of the CD4+ effector responses (92–95). Specifically, the ribonuclease activity of ω1 protein in the ES of *S. mansoni* eggs is found to be essential in inducing Th2-type response in DCs (94).

JOURNEY OF THE SCHISTOSOME EGGS

Schistosome eggs exit the host either by traversing the intestinal wall into the intestinal lumen via mesenteric vessels for *S. mansoni* and *S. japonicum*, or through migration into the vesical lumen of the bladder for *S. haematobium* (96). This egg expulsion however is mostly host-dependent as the schistosome eggs lack any motility mechanisms (97). As egg passage into the intestine is not guaranteed, about half of all the deposited eggs accidentally go to the liver (98). In order to continue transmission, the schistosome parasites employ strategies to ensure successful egg transit into the environment (98–101). Extravasation in the blood vessels is promoted by angiogenesis, endothelial activation, and fibrinolytic activity induced by schistosome eggs. The eggshell contains the enzymes enolase and glyceraldehyde-3-P-dehydrogenase (102) that act as surface binding receptors to plasminogen (103, 104). It was proposed that once it has reached the intestine, the schistosome induces granuloma formation to promote egg excretion, while at the same time preventing severe immunopathology that may otherwise affect egg release (105). It was previously noted that schistosome egg excretion is an exquisite, immune-dependent process (106).

The polarization of Th cells determines the macrophage phenotype and granuloma formation. M2 macrophage phenotype or alternatively activated macrophages are needed in effective granuloma formation and confer protection against excessive damage of the eggs during their movement across the intestinal tissue (105). M2 phenotype is promoted by IL-4/IL-13 release from Th2 cells in *S. mansoni* infection (106). This has been proven by the impaired granuloma formation during schistosome infection in T cell derived IL-4 and IL-13 deficient mice inhibiting the egg release into the intestinal lumen (107, 108). These mechanisms may suggest that Th2 immune

responses collaborate with egg-derived proteases in promoting egg release from intestinal tissues.

IMMUNOMODULATION IN THE GRANULOMA FORMATION

Unlike in intestinal granulomata, where schistosome eggs have the ability to exit into the gut lumen, the eggs in the hepatic granuloma remain trapped, with the granuloma becoming fibrotic over time. Secretions from the trapped eggs are known to stimulate the CD4+ T cells initially to release Th1-type cytokines IL-2 and IFN-γ facilitating delayed-type hypersensitivity reaction and early granuloma formation (90). This immune response gradually shifts to the Th2 phenotype with the production of IL-4, IL-5, IL-10, and IL-13 (90, 109–113). It has been shown that hepatosplenic schistosomiasis, a severe form of the disease, is associated with increased levels of Th1 cytokines TNF-α and IFN-γ, and decreased levels of Th2 cytokine IL-5 in a study done using peripheral blood mononuclear cells from patients (114). This proves that the outcome of the disease is dependent on the type of immune response elicited by the parasite within the host. In addition, *S. mansoni* eggs were shown to secrete chemokine binding protein (smCKBP) that is believed to block certain chemokines from inducing granuloma formation while preferentially altering the cellular features of the granuloma (115). Both *in vitro* and *in vivo* experiments have demonstrated that smCKBP tends to prevent the interaction of chemokines such as CXCL8 with specific cellular receptors, as well as the activation and migration of immune cells such as neutrophils (115).

Granuloma formed during prolonged Th1 response and a dampened Th2 response have been shown to display decreased size and fibrosis owing to downregulation of inflammation and of collagen deposition (116). This phenomenon might be attributed to the dominance of neutrophils infiltrating the lesion during the initiation of granuloma formation (117). Neutrophils are recruited by egg-specific proteins (118) to the core of the granuloma leading to a neutrophil-mediated inflammatory response that causes tissue damage (117). In addition, intact live eggs and soluble egg antigen (SEA) can trigger the release of neutrophil extracellular traps (NETs) within the core of the granuloma, potentially limiting the pathogenic effects of parasite eggs (119). NETs are web-like structures consisting of de-condensed chromatin and histones produced by activated neutrophils and are thought to be involved in pathogen trapping, including parasites such as *Plasmodium falciparum* (120) and *Strongyloides stercoralis* (121). A previous study has shown that eggs trapped within the mesh of NETs remain viable and were not killed, as opposed to the effect of NETs as seen in other pathogens (119). This might suggest that NETs only serve to immobilize or restrict the movement of the schistosome eggs, without adversely affecting their viability.

At a later stage of the disease, neutrophils secrete granule proteins that can degrade collagen, the major component of fibrotic granulomas, thus limiting the size of the granulomas. *S. japonicum* granuloma has neutrophils that accumulate within the core as early as 8 days post-deposition (122, 123), and at the periphery as granuloma matures (124). This implies that neutrophils have different roles in the granuloma formation depending on the time of their recruitment and their location within the lesion.

Although CD4+ T cells generally dictate the granulomatous response to the eggs, other immune cells like CD8+ T cells, B cells, M2 macrophages and eosinophils are also as important in the regulation of granuloma formation (125–128). Eosinophil infiltration in the granuloma is mediated by IL-5 and IL-13 (112, 127, 129, 130). This Th2-driven eosinophil infiltration in the granuloma stands in contrast with the early Th1 granuloma, which is dominated by neutrophils. Aside from the destructive actions directed against miracidia within trapped eggs upon their degranulation (131), eosinophils are also responsible for the polarization of the immune response to Th2 type by producing IL-4 and IL-5 (132). Granuloma in *S. mansoni* has been noted to have more eosinophils than neutrophils, which is in contrast to the neutrophil dominated granuloma seen in *S. japonicum* (133). The number of eosinophils in *S. mansoni* infection were 60.60 ± 0.47%, and 44.30 ± 0.23% of all the granuloma cells in the acute and chronic experimental infections, respectively, using murine models for both hepatic and intestinal infection (134). In an earlier experiment done using murine models for lung granuloma, results showed about 70% of the cellular population in *S. mansoni* granuloma are eosinophils at 16 days post-deposition (125).

CD4+ T cells are the primary source of IL-13 (135), the dominant Th2 cytokine responsible for the development of liver fibrosis (136). Together with IL-4, IL-13 induces macrophage expression of arginase, which then cleaves L-arginine to form L-ornithine (136). Ornithine aminotransferase then converts L-ornithine to proline, which is important in collagen production and fibrosis development (137). IL-13 also triggers the trans-differentiation of hepatic stellate cells (HSCs), one of the main sources of hepatic collagen, and plays an important role in schistosome-induced fibrogenesis (138).

The granuloma both functions as a major pathology in schistosomiasis disease and as a protective barrier between the egg and the liver tissues. Although the Th1-dominated immune response gives rise to granulomas with smaller sizes and less fibrosis, the switch to the Th2 phenotype confers some protective effects to the host (139). The granuloma functions to sequester egg secretions that can otherwise cause damage to liver tissue (139).

THE DUAL ROLE OF TGF- β

Th17 serves as a unique CD4+ T cell subset and is characterized by IL-17 production as an adaptive host mechanism in cases where both Th1 and Th2 immune responses are inappropriate for protection against the pathogen (140). IL-17 is a pro-inflammatory cytokine often seen in the pathogenesis of autoimmune diseases. In *S. japonicum*, SEAs are believed to induce a Th17 response (141) linking it to severe hepatic inflammation in schistosomiasis (142, 143). The association

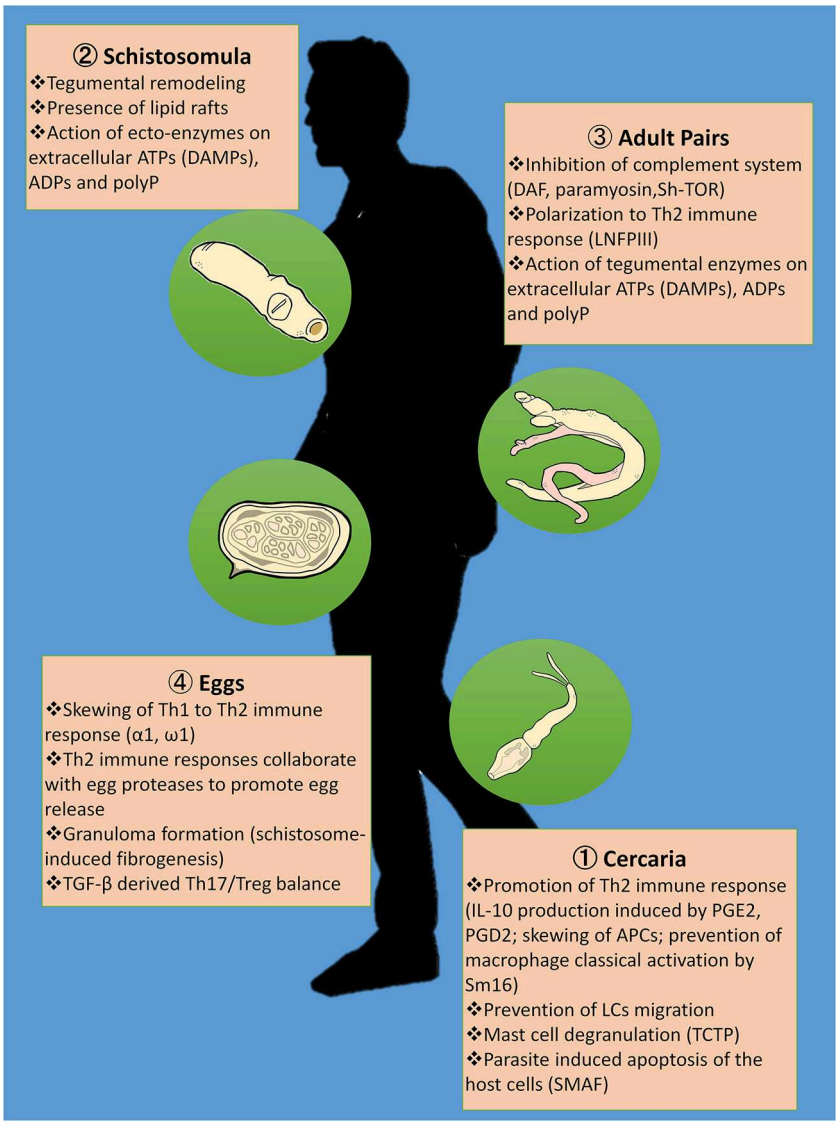

FIGURE 1 | Summary of proposed immunomodulatory strategies of the schistosome in evading the host immune responses. Skewing of Th1 to Th2 immune response is very much evident during cercarial penetration ① through IL-10 production induced by prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2), prevention of macrophage classical activation by Sm16 and disorientation of the antigen presenting cells (APCs); lodging of the adult schistosome pairs in the veins ③ through the interactions between lacto-N-fucopentose III (LNFPIII) and toll like receptors (TLRs); and egg deposition in the intestinal and liver tissues ④ through the immunomodulatory effects of the egg's excretory-secretory proteins (ES) to CD4+ effector responses including α1 and ω1. In addition, the cercariae ① are able to evade innate immunity in the skin by preventing migration of Langerhans cells (LCs), mast cell degranulation promoted by the translationally-controlled tumor protein (TCTP) homolog in schistosomes, and *Schistosoma mansoni* apoptosis-inducing factor (SMAF)-mediated host cell death. Schistosomula's ② tegumental remodeling and the presence of lipid rafts covering the parasite render them undetectable to immune responses during migration. Once the adult schistosomes ③ settle in the mesenteric veins, they become capable of evading the host complement system through the abstraction of erythrocytes' decay-accelerating factor (DAF) as seen in *in vitro* studies, the binding of human complement C8 and C9 to the schistosome's paramyosin, and the attachment of C2 to the trispanning orphan receptor of *Schistosoma haematobium* (Sh-TOR). Ecto-enzymes on the tegument of intravascular stages including the ② schistosomula and ③ adults cleave extracellular ATPs that otherwise serve as damage-associated molecular patterns (DAMPs) as well as ADPs and inorganic polyphosphates (polyPs), thereby interfering with host pro-inflammatory and prothrombotic purinergic signaling. Eggs from the schistosomes *in copula* ④ express proteases that may aid in egg egress in addition to Th2 immune responses. Granuloma formation with schistosome-induced fibrogenesis tends to limit tissue destruction brought about by egg deposition. However, disease severity is largely determined by Th17/Treg balance mediated by transforming growth factor-β (TGF-β).

between Th17 and the severity of the disease is also seen in *S. mansoni* infection as an exacerbation of granuloma in mice models is primarily directed by a Th17 response (144, 145). The role of IL-17 in granuloma formation is further proven by a decrease in the size of granulomata when anti-IL-17 neutralizing antibodies were given to infected mice (142).

Th17 differentiation is induced in mice exposed to transforming growth factor-β (TGF-β) and IL-6 (146–148).

As TGF-β is also known to induce differentiation of CD4+ T cells into forkhead box protein 3 (FoxP3)-expressing regulatory T cells (Tregs) (149), the pivotal role of TGF-β in the progress of the disease makes it one of the most important cytokines that determines disease outcome. Th17 cells promote inflammation through the production of IL-17, IL-22, and IL-23, and neutrophil recruitment (150), whereas Tregs produce the anti-inflammatory cytokines IL-10 and TGF-β (151). Surprisingly, Tregs can be transformed into Th17 cells in the presence of IL-6 (152). Interestingly, Th17 cells appear to be resistant to Tregs' suppressive effects (153, 154). Thus, the delicate balance maintained between anti-inflammatory Tregs and pro-inflammatory Th17 cells is a prime determinant in disease severity. The imbalance of Th17/Treg has been shown to be closely associated with immunopathological damage and egg granuloma formation in mouse models infected with *S. japonicum* (155).

Currently, not much is known about how the balance between the Th17/Treg immune responses modulates disease progression in schistosomiasis. It is therefore worthwhile to elucidate which mechanisms promote Treg proliferation during the chronic phase of schistosome infection when Th2 immune responses start to wane and lead to immune hypo-responsiveness.

CONCLUSION AND FUTURE DIRECTIONS

Schistosomiasis is a neglected tropical disease whose transmission has been reported in 78 countries. This parasitic disease has been a public health problem as early as 5,000 years ago upon being discovered in Egyptian mummies (156). This long relationship between humans and the schistosome parasites has enabled the latter to adopt various strategies to successfully survive inside the host. **Figure 1** shows the summary of proposed immunomodulatory armamentarium that schistosome parasites utilize in order to evade the host's immune responses, and thus facilitate infection. Understanding the mechanisms behind these immunomodulatory strategies will not only shed light on host-parasite interactions but also be useful in the development of novel treatments against the schistosome parasite.

Parasitic helminths like *Schistosoma* spp. are said to be capable of limiting intraspecific competition inside the host via concomitant immunity (157). Concomitant immunity is the production of effective anti-larval immunity that does not harm the existing adults. The adult worms might be "vaccinating" the host with cross-reactive antigens creating a barrier against new infection. This has been proven with an experimental study involving monkeys infected with adult schistosomes via surgical transplants (158). The monkeys showed resistance to cercarial challenge even though they were not exposed to any larval schistosome stages. However, another study has looked into potential mechanisms causing elimination of lung schistosomula in mice previously vaccinated with irradiated cercariae (159). Results show that the deflection of the parasites in the alveoli during migration was the reason many failed to mature in both vaccinated and unvaccinated mice, as no inflammatory reactions against the parasites have been found in the skin and lungs of the vaccinated mice (159). A better understanding of the role of immunomodulation in the early stage of schistosome infection might be the key in the production of a "true" effective anti-larval immunity against *Schistosoma*.

The 2-fold ability of the helminth worms to downregulate pro-inflammatory cytokines and skew Th1 to Th2 type immune responses has suggested their possible use in treating other illnesses such as autoimmune and inflammatory diseases, thus supporting the hygiene hypothesis (160). This hypothesis states that persons who never contract infections run the risk of developing autoimmune diseases, as infections facilitate the development and regulation of the immune system (161). Therefore, immunomodulatory molecules elaborated in response to the schistosome parasite can serve as potential tools to control overt immune responses.

Experimental studies have demonstrated the immunomodulatory effects of schistosome infection on arthritis (162–164), type 1 diabetes (165–168), Graves' disease (169), and airway allergies (170, 171). The therapeutic potentials of immunomodulatory molecules such as smCKBP might be used as selective manipulators of the immune system to prevent immune-mediated diseases (115). The schistosome-derived carbohydrate LNFPIII might be useful in treating type 2 diabetes as its administration in mice has improved glucose tolerance and insulin sensitivity (172), and in psoriasis as it induces Th2 immune response, and subsequent amelioration of skin lesions (173). Purified cystatin from *S. japonicum* has been shown to reduce inflammatory parameters and decrease the severity of trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice, thereby demonstrating its potential therapeutic use in inflammatory bowel diseases (174). Taking advantage of these adaptive mechanisms of the schistosome parasite thus offers promise in the management of various autoimmune and inflammatory conditions. More immunomodulatory molecules and their interactomes and mechanisms need to be identified and characterized to develop effective drugs to achieve this end.

AUTHOR CONTRIBUTIONS

JA, VM, and PR contributed on the conception of the review paper. JA wrote the first draft of the manuscript. JA, VM, and PR wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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To the Skin and Beyond: The Immune Response to African Trypanosomes as They Enter and Exit the Vertebrate Host

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African trypanosomes are single-celled extracellular protozoan parasites transmitted by tsetse fly vectors across sub-Saharan Africa, causing serious disease in both humans and animals. Mammalian infections begin when the tsetse fly penetrates the skin in order to take a blood meal, depositing trypanosomes into the dermal layer. Similarly, onward transmission occurs when differentiated and insect pre-adapted forms are ingested by the fly during a blood meal. Between these transmission steps, trypanosomes access the systemic circulation of the vertebrate host *via* the skin-draining lymph nodes, disseminating into multiple tissues and organs, and establishing chronic, and long-lasting infections. However, most studies of the immunobiology of African trypanosomes have been conducted under experimental conditions that bypass the skin as a route for systemic dissemination (typically *via* intraperitoneal or intravenous routes). Therefore, the importance of these initial interactions between trypanosomes and the skin at the site of initial infection, and the implications for these processes in infection establishment, have largely been overlooked. Recent studies have also demonstrated active and complex interactions between the mammalian host and trypanosomes in the skin during initial infection and revealed the skin as an overlooked anatomical reservoir for transmission. This highlights the importance of this organ when investigating the biology of trypanosome infections and the associated immune responses at the initial site of infection. Here, we review the mechanisms involved in establishing African trypanosome infections and potential of the skin as a reservoir, the role of innate immune cells in the skin during initial infection, and the subsequent immune interactions as the parasites migrate from the skin. We suggest that a thorough identification of the mechanisms involved in establishing African trypanosome infections in the skin and their progression through the host is essential for the development of novel approaches to interrupt disease transmission and control these important diseases.

Keywords: African trypanosomiasis, *Trypanosoma brucei*, skin, transmission, innate immunity, neglected tropical disease

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INTRODUCTION

African trypanosomiasis has historically been the cause of large outbreaks of human disease, likely contributing to the deaths of millions of people across sub-Saharan Africa in the early twentieth century (1, 2) and inflicting substantial economic damage on the African agriculture industry to this day (3, 4). African trypanosomes include an array of vector-borne, single cell hemoflagellate protozoa (order *Kinetoplastida*), although three species cause major disease: *Trypanosoma brucei*, *T. congolense*, and *T. vivax. Two* subspecies of *T. brucei*, *T. b. rhodesiense,* and *T. b. gambiense*, cause human African trypanosomiasis (HAT), also known as sleeping sickness (5–7), with more than 57 million people at risk of infection (6, 8). *T. congolense*, *T. vivax*, and *T. brucei* are also the most significant contributors to disease in livestock animals (animal African trypanosomiasis or AAT). Classically, the trypanosome lifecycle starts with the tsetse fly (*Glossina* spp.) depositing an inoculum of metacyclic trypomastigotes in the skin when taking a blood meal (9–11). Following intradermal (i.d.) inoculation of metacyclic forms, the parasites differentiate into long-slender trypomastigotes that are proliferative and able to establish patent infections in the vertebrate host. However, the timing and the mechanisms controlling these events remain unclear (3, 12, 13). From the initial site of infection, the proliferative long-slender form trypanosomes travel to the local draining lymph nodes *via* afferent lymphatic vessels before disseminating systemically and establishing a patent infection in the bloodstream (14–17). African trypanosomes (and *T. brucei* in particular) also actively colonize multiple tissues in the vertebrate host, including the skin. Skin-dwelling parasites functionally and behaviorally adapt to their microenvironment, allowing them to thrive and persist (18, 19). These recent studies demonstrate that there is a previously underappreciated heterogeneity in the population of parasites residing within the vertebrate host, with important implications for understanding the biology of trypanosomes and the way in which the host responds to infection. The presence of trypanosomes in the skin has been demonstrated in both animal models of infection and human clinical samples, suggesting that it is a central aspect of transmission. Nonetheless, the mechanisms deployed by trypanosomes to inhabit and migrate from the cutaneous environment, and the interplay between resident skin cells (including immune cells), and trypanosomes during the onset of the infection, remain largely unexplored. In this review, we aim to (i) highlight current knowledge on trypanosome establishment of infection in the skin; (ii) examine the interactions between the host immune system and trypanosomes in the skin; (iii) explore the mechanisms of trypanosome migration from the skin toward systemic infection and further transmission; and finally (iv) discuss the potential of novel therapeutic and intervention strategies being developed as a consequence of these studies.

Skin as the Initial Barrier—From Immune Response to Systemic Dissemination

Upon infection, metacyclic trypomastigotes must circumvent several environmental challenges in order to develop into the proliferative long-slender form trypomastigotes. This series of events ultimately leads to parasite dissemination in the host bloodstream but involves interactions between the developmental stages of the parasite, the host cells in the dermis, and the immune cells recruited to the site of infection. Mammalian skin is a large, highly complex organ that acts as a protective barrier between the internal components of the host and the external environment (**Figure 1**) (20, 21). The mechanism by which the skin protects the host is not simply through providing a physical barrier, but also the collection of immune cells, biological factors, layers of tissue, and networks of lymphatic and blood vessels (21–23). The three main components of the skin are the epidermis, dermis, and subcutaneous layer, each containing various immune cells involved in innate responses, inflammation, and surveillance (**Figure 1A**) (21, 24). The dermis is primarily connective tissue produced by dermal fibroblasts. Local immune responses generated within the tissue are initiated by dermal macrophages, dermal dendritic cells, natural killer (NK) cells, mast cells, αβ/γδ T cells, and natural killer T (NKT) cells (25, 26). The skin also contains numerous blood and lymphatic vessels, nerves, and (in humans but not mice) sweat glands (23, 27, 28). Together, these layers create a highly organized body compartment that represents a strong physical and biological barrier to pathogens and systemic infections.

The Tsetse Fly Vector, the Feeding Bite, and the Site of Infection

Within an infected tsetse fly, *T. brucei* undergoes a range of complex developmental stages (29). When first taking a blood-meal from an infected mammalian host, parasites are ingested, and become proliferative procyclic trypomastigote forms in the midgut of the tsetse fly. These procyclic trypomastigotes divide rapidly before transforming into mesocyclic forms in the alimentary tract that then invade the salivary glands and transform into the rapidly proliferating epimastigotes. Tsetse saliva provides an environment that promotes trypanosome adherence to epithelial surfaces, the initiation of binary fission, and also triggers their transformation into mammal-infective metacyclic trypomastigotes (29–31). Without the presence of these salivary components, metacyclic trypomastigotes have reduced infectivity (32). Following an infected tsetse fly feeding on a mammalian host, trypanosomes are deposited into the dermis of the host skin (11). During this process, the tsetse fly proboscis inflicts significant trauma on the skin and associated tissues, while also introducing a concoction of active compounds via the saliva (33). In humans, a trypanosome-filled lesion known as a chancre often develops 5–15 days post-inoculation, which comprises indurated and inflamed patches of skin (34, 35). Similar lesions also occur in goats and cattle (36). The development of a chancre may be related to several components of tsetse saliva that have been shown to affect inflammation at the site of infection, including 5′nucleotidase-related (5′Nuc), tsetse thrombin inhibitor (TTI), and both thrombin serine protease, and esterase inhibitors (30, 37–40). This altered immune state is characterized by elevated interleukin IL-4 and IL-10 production

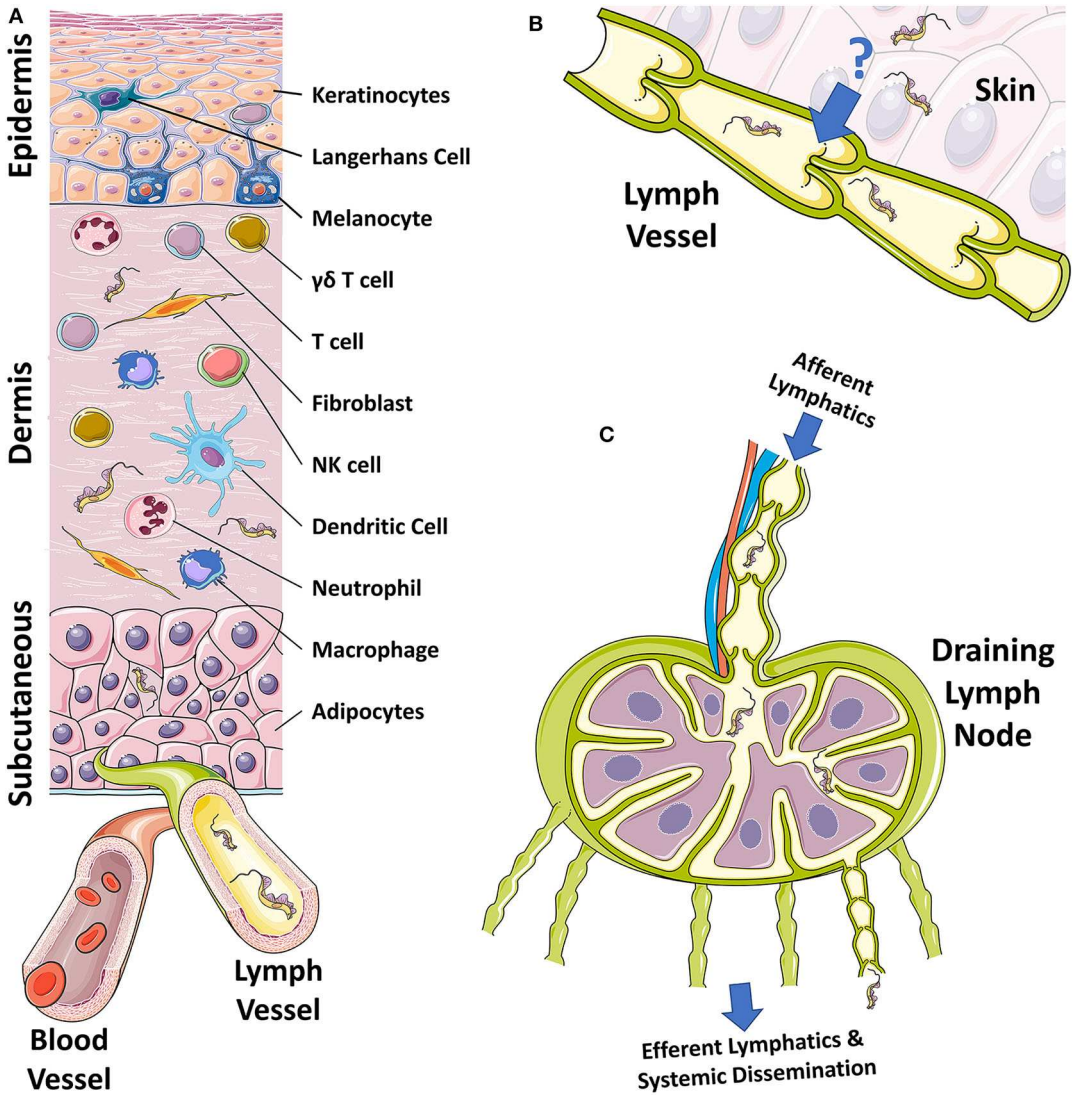

FIGURE 1 | The skin, draining lymphatics, and lymph nodes. **(A)** Diagram of the cellular composition of the epidermal, dermal, and subcutaneous layers of mammalian skin. The outermost epidermal layer consists of a layer of corneocytes above a layer of keratinocytes. These cells manage the tight junctions and the stratum corneum. Langerhans cells and intraepithelial T cells survey the epidermis for antigen to be presented. The central dermal layer contains fibroblasts that produce extracellular matrix proteins to provide structural support and elasticity. Immune responses are initiated by dermal macrophages, dermal dendritic cells, NK cells, and T cells. The inner subcutaneous layer primarily consists of adipocytes. Local lymphatic and blood vessels allow for the trafficking of cells, proteins, and waste. The initial tsetse fly bite injects trypanosomes into the dermis. From the dermis, the parasites exhibit tropism that leads to migration toward the afferent lymph vessels in the skin disseminating to the blood and other regions of the body. **(B)** The mechanism behind directional migration of trypanosomes from the skin to the lymphatics is unknown. Parasites may be responding to an unreported chemical cue in a chemotactic manner and they may crawl along lymph vessels, access open junctions, or are drawn into the lymphatics through hydrodynamic flow force and pressure. **(C)** Afferent lymphatic vessels in the skin allow for the drainage of leukocytes and antigen into the draining lymph node. Lymph, containing activated T and B cells, plasma cells, and antibody, passes into the medullary sinus, before exiting via efferent lymphatic vessels. Trypanosomes enter the draining lymph nodes, causing lymphadenopathy, and exit via the efferent lymphatics. Systemic dissemination of the host is reached via the main lymphatic ducts.

(30, 32, 40–42). Murine models of infection have also shown that tsetse saliva suppresses T and B cell responses systemically, skewing the host toward a Th2 immune environment, leading to increased IL-4 and IL-10, alongside decreased interferon-γ (IFN-γ) titers, in the draining lymph nodes of infected mice. There is also an associated immunoglobulin (Ig) IgG1 and IgE antibody response. In addition, bites from tsetse flies infected with trypanosomes showed significantly reduced thrombin inhibition and less anticoagulation compared to bites by naïve tsetse flies. This results in a more prolonged feeding period that contributes to an increased likelihood of parasite transmission (9). Together, these anti-inflammatory and anti-coagulative processes act to increase the efficiency of parasite transmission from the fly vector to the mammalian host (32),

leading to the induction of microenvironmental conditions more suitable for metacyclic trypomastigotes. This facilitates their differentiation into proliferative long-slender trypomastigotes. In this context, it appears likely that metacyclic trypomastigotes are equipped with various molecular tools (e.g., secreted virulence factors) to initiate tissue colonization events and overcome the acute response elicited by skin-resident and recruited immune cells. This leads to a scenario in which the acute immune response at the site of infection may act as a bottleneck that selects for more infectious parasites. Consequently, the route of infection could potentially shape downstream interactions and responses in other body compartments, affecting parasitaemia and longer-term infection dynamics.

Influence of the Infection Route for Trypanosome Dissemination

Despite the skin being the first point of contact between metacyclic trypomastigotes and the vertebrate host, the importance of the skin-stage of disease in trypanosome infections has largely been overlooked in experimental studies. The majority of prior experiments have examined infections initiated *via* needle challenge, predominantly by intraperitoneal (i.p.) or intravenous (i.v.) routes (**Table 1**). However, a small number of comparative experiments have revealed that inoculation routes can substantially affect outcome (43, 44). For example, it has been shown that the percentage of BALB/c mice with detectable parasitemia after infection by *T. brucei* differed between animals infected i.p. vs. i.d. This study also found that the proportions of mice displaying detectable parasitemia were significantly reduced in the i.d. infected mice compared to i.p. infected mice, and that i.d. infected mice were 100-fold less susceptible to trypanosome infection than i.p. infected mice in a dose-dependent manner (43). Moreover, the impact of infection route on infectivity also differs between trypanosome species (44). For example, *T. congolense* infected intramuscularly (i.m.) had an earlier onset of parasitemia compared to those infected *via* an i.d. route. However, animals in which *T. brucei* parasites were administered i.p. led to an earlier onset of parasitemia than both i.m. and i.d. administration. These observations suggest that parasites face tissue-specific challenges in the skin (e.g., resident immune cells or nutrient availability) that delineate their capacity to disseminate systemically within the vertebrate host (44).

In addition to infection route, inoculum dose has also been shown to influence the outcome of trypanosome infection (44). For example, goats infected i.d. with *T. congolense* showed a delay in the onset of a local skin reaction (chancre formation) as the inoculum dose decreased (66), with a concomitant decrease in the size of the chancre. Consistent with these observations, a similar effect was reported for BALB/c mice infected i.d. with *T. brucei*, showing reduced infection rates at lower doses compared to higher doses (43). In this study, all animals infected with doses ranging from 1×10^5 to 1×10^4 parasites developed detectable parasitemia. Conversely, doses of 1×10^2 parasites led to no patent infections and 1×10^3 parasites resulted in only 50% of the animals developing a patent infection. In contrast, all the mice infected with 1×10^2 parasites i.p. developed detectable parasitemia at the same time point, indicating that the skin poses a significant barrier for systemic dissemination and infection dynamics. These results suggest that there is a minimal infective parasite dose able to survive the initial challenge mounted by the local immune response in the skin.

The dose and route of infection has been shown to affect the dynamics of several protozoan infections, including the related trypanomastid *Leishmania*. These parasites are similarly transmitted to the skin of the mammalian host through the bites of female sand fly vectors (67), although they differ from extracellular African trypanosomes in that the inoculated lifecycle stage invades mammalian cells and replicates intracellularly. Upon feeding, the sand fly regurgitates metacyclic promastigote forms into the skin that are then phagocytosed by host macrophages. Promastigotes then develop into amastigote forms and replicate within the host cell. Subcutaneous (s.c.) injections of *Leishmania major* and *L. tropica* in mice result in a lower systemic parasite burden and increased protective immunity compared to i.p. and i.d. infections (68, 69). An increased Th1-associated resistance to *Leishmania* infection was induced in BALB/c mice following a low dose of parasite inoculum administered either i.d. or s.c. (69, 70), whereas a high inoculum induced a more Th2-skewed immune response that led to higher susceptibility and systemic parasite burden. These observations in related parasites highlight the importance of the initial parasite dose and site of infection for disease outcome. More importantly, these initial interactions might favor further dissemination (e.g., by infecting recruited immune cells) or the formation of quiescent parasite foci relevant for diagnostics and infection recrudescence upon treatment. It is likely that there are similar effects occurring during the initial skin stages of African trypanosome infections, although the factors determining parasite survival and migration remain to be fully explored.

Immunity to African Trypanosomes in the Skin

African trypanosomes exist entirely extracellularly within the mammalian host and are constantly exposed to the host innate and adaptive immune systems. Previous studies examining systemic immune responses in mice using artificial inoculation routes (primarily i.p. and i.v.) have found numerous components that are vital to controlling the initial stages of trypanosome infection, including macrophages, monocytes, dendritic cells, neutrophils, and NK cells (**Figure 2**) (71–75). The early immune response (<2 weeks) is characterized by an induction of a strong pro-inflammatory profile, including the expression of IFN-γ, tumor necrosis factor (TNF), IL-6, and the production of nitric oxide (NO) (3, 76–82). An adaptive B cell response is also elicited, leading to the production of antigen-specific antibodies that target the immunodominant variant surface glycoprotein (VSG) at the parasite surface. However, the artificial inoculation routes used in these studies overlook the events that occur in the skin early during infection. Through understanding the processes involved in establishing infection in the mammalian

TABLE 1 | Susceptibility of various animal models to different trypanosome strains represented by the proportion of animals displaying a patent infection, depending on route and dose.

Trypanosome species and strain	Mammalian host species	Animal strain/breed	Inoculation route	10^6 dose	10^5 dose	10^4 dose	10^3 dose	10^2 dose	References
T. b. brucei, strain 10–26	Mouse	BALB/c	i.p.	–	100%	100%	100%	100%	(43)
T. b. brucei, strain 10–26	Mouse	BALB/c	i.d.	–	100%	100%	50%	0%	
T. congolense, *Trans Mara*, TC13	Mouse	BALB/c	i.p.	–	–	100%	100%	100%	
T. congolense, *Trans Mara*, TC13	Mouse	BALB/c	i.d.	–	–	100%	100%	0%	
T. b. brucei, strain 10–26	Mouse	C57BL/6	i.p.	–	100%	100%	100%	100%	
T. b. brucei, strain 10–26	Mouse	C57BL/6	i.d.	–	100%	100%	50%	0%	
T. congolense, *Trans Mara*, TC13	Mouse	C57BL/6	i.p.	–	100%	100%	100%	100%	
T. congolense, *Trans Mara*, TC13	Mouse	C57BL/6	i.d.	–	–	100%	100%	0%	
T. b. brucei, KETRI 2710	Mouse	Swiss white	i.p.	–	–	100%	–	–	(44)
T. b. brucei, KETRI 2710	Mouse	Swiss white	i.v.	–	–	100%	–	–	
T. b. brucei, KETRI 2710	Mouse	Swiss white	i.m.	–	–	100%	–	–	
T. b. brucei, KETRI 2710	Mouse	Swiss white	s.c.	–	–	100%	–	–	
T. b. brucei, KETRI 2710	Mouse	Swiss white	i.d.	–	–	83%	–	–	
T. congolense, KETRI 2765	Mouse	Swiss white	i.p.	–	–	67%	–	–	
T. congolense, KETRI 2765	Mouse	Swiss white	i.v.	–	–	50%	–	–	
T. congolense, KETRI 2765	Mouse	Swiss white	i.m.	–	–	100%	–	–	
T. congolense, KETRI 2765	Mouse	Swiss white	s.c.	–	–	100%	–	–	
T. congolense, KETRI 2765	Mouse	Swiss white	i.d.	–	–	100%	–	–	
T. (Dutonella) vivax, IL 1392	Mouse	CD-1	i.p.	–	–	–	–	100%	(45)
T. (Dutonella) vivax, IL 1392	Mouse	CD-1	s.c.	–	–	–	–	100%	
T. congolense (GVR 12/1),	Mouse	CD-1	i.v.	100%	100%	100%	100%	–	(46)
T. congolense, *Trans Mara*, TC13	Mouse	BALB/c	i.p.	–	–	–	100%	100%	(47)
T. congolense, *Trans Mara*, TC13	Mouse	BALB/c	i.d.	–	–	–	0%	0%	
T. congolense, IL 3274	Mouse	Swiss white	i.v.	100%	100%	100%	100%	100%	(48)
T. b. rhodesiense, EATRO 1886	Cattle	Boran	i.v.	–	100%	–	–	–	(49)
T. vivax, *ETBD-1/ETBS 1*	Cattle	Zebu	i.v.	100%	–	–	–	–	(50)
T. vivax, Y58	Cattle	Zebu	i.v.	–	–	100%	–	–	(51)
T. congolense, TREU 112	Cattle	Holstein	i.v.	100%	–	–	–	–	(52)
T. evansi, Olmisor isolate	Goat	East African	i.v.	–	100%	–	–	–	(53)
T. congolense, Ea-Tc, IL 1180	Goat	West African Dwarf	i.d.	–	–	100%	–	–	(54)
T. congolense, ITC 84	Goat	West African Dwarf	i.d.	–	–	100%	–	–	
T. congolense, IL 957	Goat	East African	s.c.	–	100%	–	–	–	(55)
T. congolense, IL 958	Goat	Galla	s.c.	–	100%	–	–	–	
T. congolense, UTRO 170491-B	Goat	Kigezi	i.v.	–	100%	–	–	–	(56)
T. congolense, UTRO 170491-B	Goat	Mubende	i.v.	–	100%	–	–	–	
T. congolense, UTRO 170491-B	Goat	Small East African	i.v.	–	100%	–	–	–	
T. congolense	Goat	Galla	i.v.	–	100%	–	–	–	(57)
T. congolense	Goat	East African	i.v.	–	100%	–	–	–	
T. congolense	Goat	Saanaen	i.v.	–	100%	–	–	–	
T. congolense	Goat	Saanen x Galla	i.v.	–	100%	–	–	–	
T. congolense	Sheep	Merino	i.v.	–	100%	–	–	–	
T. congolense	Sheep	Blackhead Persian	i.v.	–	100%	–	–	–	
T. congolense	Sheep	Red Masai	i.v.	–	100%	–	–	–	
T. brucei, CT 70	Sheep	Yankassa rams	s.c.	100%	–	–	–	–	(58)
T. vivax, CT 128	Sheep	Yankassa rams	s.c.	100%	–	–	–	–	
T. congolense, GT 12	Sheep	Yankassa rams	s.c.	100%	–	–	–	–	
T. brucei, Strain 8/18	Sheep	West African Dwarf	i.v.	100%	–	–	–	–	(59)

(Continued)

TABLE 1 | Continued

Trypanosome species and strain	Mammalian host species	Animal strain/breed	Inoculation route	10^6 dose	10^5 dose	10^4 dose	10^3 dose	10^2 dose	References
T. vivax, Zarkwai/84/ NITR/11.1	Sheep	Uda	i.v.	–	100%	–	–	–	(60)
T. congolense, Ea-Tc, IL 1180	Sheep	Djallonke	i.d.	–	–	100%	–	–	(54)
T. congolense, ITC 84	Sheep	Djallonke	i.d.	–	–	100%	–	–	
T. b. brucei	Rabbit	New Zealand white	i.p.	100%	–	–	–	–	(61)
T. b. brucei	Rabbit	Chinchilla White	i.p.	100%	–	–	–	–	
T. rhodesiense, EATRO 1886	Rabbit	New Zealand white	i.v.	–	–	100%	–	–	(62)
T. b. gambiense, MBA ITMAP 1811	Monkey	Vervet	i.v.	–	–	–	100%	–	(63)
T. b. brucei, GUTat 1	Monkey	Vervet	i.v.	–	–	100%	–	–	(64)
T. b. rhodesiense, KETRI 2537	Monkey	Vervet	i.v.	–	–	100%	–	–	(65)

i.p., intraperitoneal; i.d., intradermal; i.v., intravenous; i.m., intramuscular; s.c., subcutaneous.

host and establishing how the immune system interacts with the parasites in the initial stages, skin-targeted research could provide important information on how the disease progresses within the host. This additional information may reveal methods to develop novel methods for controlling disease transmission in humans and animals. It is also likely that the immune responses in the skin have wider impacts on the systemic infection, similar to those observed in *Leishmania* infections (69, 70) and recent data have shown that there is a population of African trypanosomes present in the skin of the host (11, 18, 83). These parasites are integral to transmission, but their persistence suggests that the parasites can avoid or co-opt the immune response in the skin. Understanding how this is achieved could lead to methods to limit this population, reducing transmission. This skin-dwelling population also presents issues for new therapeutics targeting African trypanosomes that have been developed for parasites in the bloodstream and CNS. Although there has been little work to date on skin immune responses during trypanosome challenge, inferences can be made from systemic studies and related parasites, in addition to the small number of studies that have been performed.

Neutrophils and Natural Killer (NK) Cells—the First Responders

It is likely that some of the earliest cellular responders to trypanosome inoculation in the skin are neutrophils, NK cells, and NKT cells (3, 84, 85). Neutrophils are some of the most ubiquitous leukocytes in the human immune system and are involved in the killing of many pathogens (including protozoa). They act through phagocytosis, the release of reactive oxygen species, and neutrophil extracellular traps (NETs) (86–89). They are also important mediators of tissue repair and wound healing, producing various pro-inflammatory cytokines that include transforming growth factor-β (TGF-β), IL-4, IL-12, and IL-13 (88). They may also potentially release IFN-γ, although it is unclear whether neutrophils are a true source of IFN-γ, especially in humans (90). As such, our understanding of these mechanisms relies heavily on murine data that may not be applicable to human mechanisms of immunity. In these model experiments, neutrophils have been shown to be the primary responders to tsetse fly bites and are recruited to the dermal bite site within 4.5 h (85). At the same time as neutrophil recruitment, there is an induction of pro-inflammatory IL-1β and IL-6, as well as anti-inflammatory IL-10 (85), from unidentified sources. Neutrophils may also produce trypanolytic antimicrobial peptides, such as cathelicidins and defensins (91), although this early neutrophil response does not appear to contribute to trypanosome killing in the skin (85).

Cytotoxic NK and NKT cells are also commonly employed during the earliest periods of pathogen infection. However, the roles of NK cells during trypanosome infection are poorly understood and investigation is limited to systemic studies. NK cell-deficient mice infected with *T. congolense* are unable to control the levels of parasitemia due to lower levels of IFN-γ and TNF, leading to rapid onset of death due and uncontrollable parasitemia (92). This lethal phenotype was rescued when NK cells were transferred into the NK cell-deficient mice prior to infection. Moreover, mice infected i.p. with *T. congolense* show a systemic recruitment of NK cells that are thought to provide early production of IFN-γ and TNF in the blood, spleen, lungs, and liver (92). NK cell activity in trypanotolerant strains of mice (strains that display reduced clinical disease when infected with trypanosomes compared to susceptible counterparts) infected with *T. congolense* has been suggested to be due to an increase in the production of IFN-γ during infection (72). The activation of neutrophils, NK cells, and NK/T cells in the skin following exposure to trypanosomal pathogen-associated molecular patterns (PAMPs) also results in the production of IFN-γ and TNF that can induce the activation of classically activated macrophages (93). In this context, we speculate that recruitment of NK cells in the skin also provide early protective immunity in trypanotolerant hosts, utilizing pro-inflammatory cytokines to promote further immune activation and parasite killing. Studies regarding the spatio-temporal recruitment of immune effectors to the skin during *T. brucei* infection might shed lights into these processes.

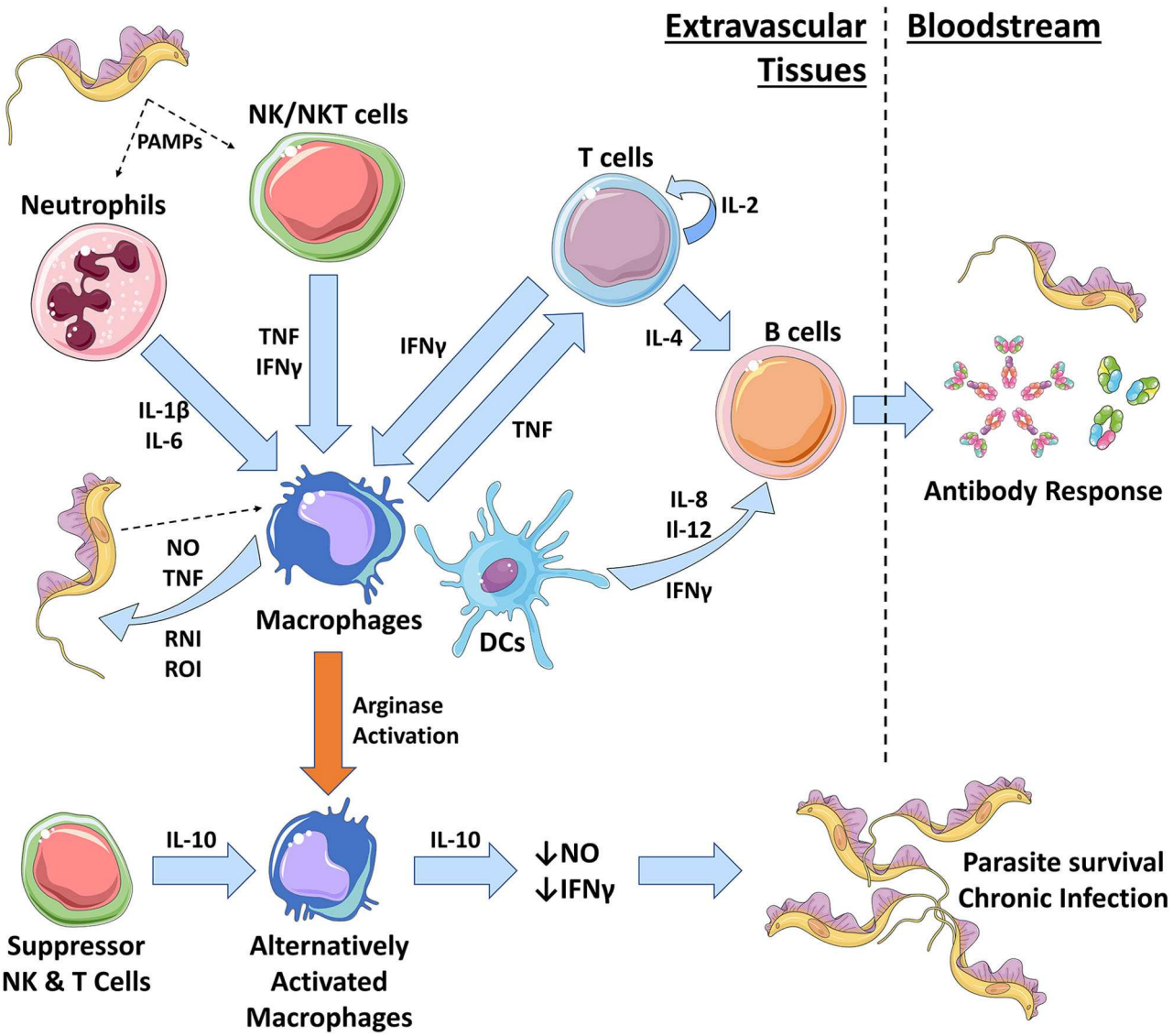

FIGURE 2 | The role of innate immune cells during African trypanosomiasis. During early trypanosome infection, a strong Th_1 immune response is initiated by the host. In the skin, neutrophils and NK cells are the first to respond to trypanosomal pathogen-associated molecular patterns (PAMPs), such as VSG and CpG DNA. Neutrophils are heavily involved in repairing the initial wound caused by the tsetse fly bite and also produce pro-inflammatory IL-1β and IL-6. NK cells produce pro-inflammatory TNF and IFN-γ that results in the classical activation of pro-inflammatory macrophages (Mθ) via iNOS activation. Macrophages can also be activated through interactions with trypanosomal PAMPs. Classically activated macrophages produce further pro-inflammatory molecules, including TNF, nitric oxide (NO), and reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs). These chemicals can directly kill trypanosomes in extravascular spaces and tissues and allows for parasite control. Macrophage secretion of TNF can also recruit and activate T cells which self-renew via autocrine IL-2 secretion. T cells produce IFN-γ to further activate macrophages and IL-4 to activate B cells. Macrophages and dendritic cells (DCs) will further activate B cells during a Th_1 response, via IL-6, IL-12, and IFN-γ, to promote the production of antibodies that can target the VSG trypanosomes, inducing waves of parasite clearance in the bloodstream. However, antigenic variation hinders the effective clearance of trypanosome populations. Macrophages can also become alternatively activated, resulting in a Th2 immunosuppressive response. Cytokines such as IL-10, IL-4, and TGF-β initiate this type of response by promoting arginase activation in macrophages. As a result, alternatively activated macrophages produce immunosuppressive IL-10 and suppress production of trypanostatic NO and IFN-γ. This promotes parasite growth and survival, leading to a chronic infection.

Similar to African trypanosomes, *Leishmania* spp. are transmitted into the subdermal layer of the skin by female phlebotomine sand flies (67, 94). In this case, it has been shown that neutrophils in the skin are the initial responders to infection and phagocytose *Leishmania* metacyclic promastigotes (95). Two-photon intravital imaging has shown that 40–60 min post-infection, a rapid early recruitment of neutrophils is induced at the site of infection in the skin following sand fly feeding (96). Here, the neutrophils occupy the epidermis in large numbers where they directly kill promastigotes using NETs (86,

97) and active phagocytosis of parasites (96). However, there is substantial evidence that promastigotes can persist within neutrophils, escaping effective killing, and potentially taking advantage of neutrophils as a means to continue their life cycle within the mammalian host (98, 99). The promastigotes in the infected neutrophils are in turn phagocytosed by macrophages and dendritic cells where they develop into the amastigote form and replicate (94, 95). This neutrophil recruitment response is similar to that observed following an infected tsetse fly bite (85). However, while direct killing by neutrophils has been shown with *Leishmania* parasites, it has not been shown during African trypanosome infections. One hypothesis is that the rapid recruitment of neutrophils during trypanosome infection occurs in response to the tissue damage inflicted by the tsetse fly and is required for wound repair. In this regard, the initial recruitment of neutrophils upon *T. brucei* infection in mice (*via* i.p.) is prolonged after the initial peak of parasitemia. This is thought to contribute to the suppression of NK, NK/T cells, and both T and B lymphocytes in the spleen at later stages of infection (100). This may be through disruption of the splenic microarchitecture due to significant pro-inflammatory responses. How these immunosuppressive mechanisms relate to early infection in the skin, as well as the activation of the skin-resident immune population, remain unknown and should therefore be an important area for further investigation.

Macrophages—the Big Players

The skin contains an abundance of macrophages with the potential to combat infection but again, little is known about the role of skin-resident macrophages during trypanosome infection. During systemic infections, macrophages are considered to play an essential role in combating African trypanosome infections in the mammalian host and are central to mediating the immune response in the extravascular tissues (**Figures 2**, **3**) (3, 74, 101–110). After infection, trypanosome associated PAMPs trigger the activation of these innate mononuclear phagocytes *via* interactions with pattern recognition receptors (PRRs) on the surfaces of host immune cells. These are triggered by parasite-derived molecules such as CpG DNA (recognized by TLR-9) and soluble glycosylphosphatidyl inositol (GPI)-anchored VSG (recognized by scavenger receptor PRRs) (36, 74, 84, 103, 105, 108, 111–115). The release of soluble VSG further stimulates immune cells in a type 1-dependent manner (112). These trigger signaling pathways that lead to the acquisition of a classically activated phenotype in macrophages (and the release of pro-inflammatory cytokines such as IFN-γ and TNF), which is important for quickly controlling invading trypanosomes (**Figure 3**) (36, 84, 103, 105, 108, 112–114).

The strong pro-inflammatory type 1 (Th1) immune response initiated during the early onset of infection, characterized by elevated levels of IFN-γ and IL-2, leads to an increase in macrophage numbers in the spleen, liver, and bone marrow (106). During clinical disease, macrophage migrating inhibitory factor (MIF) mRNA levels are also increased in the blood of infected patients (116). This could explain the elevated levels of recruited macrophages observed in peripheral immune organs. Macrophages and liver-resident Kupffer cells phagocytose trypanosomes that are opsonized by parasite-specific Ig. These cells are aided by various soluble factors derived from the host complement system (106, 117–119). Experiments using *T. brucei*-infected mice have shown that Kupffer cells in the liver are involved in most of the parasite clearance that occurs *via* complement and antibody-mediated phagocytosis (120). Classically activated macrophages can also utilize the inducible nitric oxide synthase (iNOS) enzyme to produce highly reactive and toxic nitric oxide (NO) *via* the L-arginine metabolic pathway (**Figure 3**) (121, 122). Additionally, these mononuclear phagocytes can produce further pro-inflammatory cytokines such as TNF, IL-1, IL-6, IL-8, and IL-12 (93). Interestingly, several pro-inflammatory cytokines have been shown to display potent trypanostatic activities, further highlighting the importance of these cytokines during the onset of the infection (3, 72, 84, 123–130). One such trypanostatic effect is believed to be mediated by NO as experimental infections with *T. congolense* and *T. brucei* have shown that NO production can inhibit trypanosome growth and specifically control the first wave of parasitemia (131, 132). However, the role of NO is contentious as *in vivo* studies have reported that NO readily binds to hemoglobin and interacts with red blood cells (76–78). This would mean that NO would be quickly quenched in the bloodstream, removing its inhibitory effect. However, NO may still be effective in the microenvironment of extravascular spaces in which hemoglobin is much reduced or absent, such as the skin (133). Importantly, not all interactions with cytokines appear to be detrimental to trypanosomes. Trypanosomes are known to secrete trypanosome lymphocyte triggering factor (TLTF) that can trigger the production of IFN-γ from CD8+ T cells (134, 135), inducing a potent classically-activated macrophage response. This seemingly contradictory effect may suggest that African trypanosomes can benefit from this specific T cell interaction for survival, or alternatively, that TLTF might play an important role in inducing the development of a more immunotolerant environment for the parasite to sustain survival. This remains speculative but merits further investigation.

The induction of type 2 macrophages is a prominent feature of the immunosuppression that occurs during chronic *T. brucei* infections. Studies have shown that trypanosomes trigger a switch in macrophage activation from pro-inflammatory (or classically-activated) cells to a more anti-inflammatory (or alternatively activated) state (74, 104, 110, 136, 137). This switch in macrophage character skews the host immune response from Th1 to Th2, resulting in an anti-inflammatory environment that promotes parasite survival in the host (138). The implications for this profound switch in immunological state are a matter of active research but the process may contribute to reducing the deleterious effects on the host of sustained inflammation, as well as favoring tissue repair and regeneration (93, 139–143). Macrophages can become alternatively activated through stimulation by macrophage colony-stimulating factor (CSF-1), IL-4, IL-10, IL-13, and TGF-β (**Figure 3**) (93, 144–146). One of the main hallmarks of alternatively-activated macrophages (AAM) is the expression of arginases upon stimulation, which in turn compete with iNOS and induce ornithine and urea

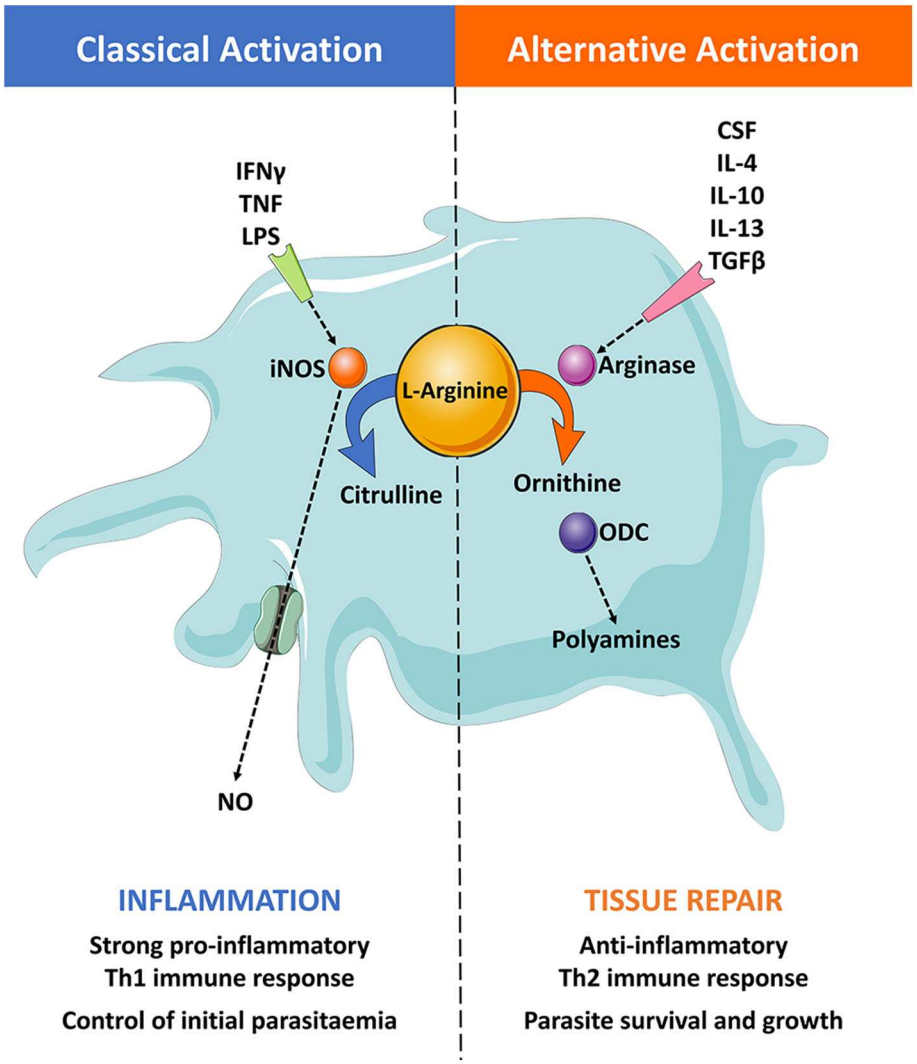

FIGURE 3 | The roles of classically and alternatively activated macrophages. (Left) Pro-inflammatory stimuli, such as IFN-γ, TNF-α, and LPS, classically activates macrophages. Classically activated macrophages induce the expression of the inducible nitric oxide synthase (iNOS) enzyme, which catabolizes the substrate L-arginine to produce NO and citrulline. This results in a pro-inflammatory Th1 immune response that can effectively control the initial parasitaemia peak. (Right) Anti-inflammatory stimuli, such as CSF, IL-4, IL-10, IL-13, and TGF-β, alternatively activate macrophages. These induce the expression of the arginase enzyme that catabolizes the substrate L-arginine to produce ornithine. The enzyme ornithine decarboxylase (ODC) catalyzes the breakdown of ornithine to produce polyamines, resulting in an anti-inflammatory Th2 immune response and tissue repair. This type of response typically leads to an immune environment that promotes trypanosome growth and survival.

production *via* the L-arginine pathway instead of NO and citrulline (147).

A recent study has also shown that *T. brucei* can actively skew macrophage and glial cell activation by secreting metabolites that suppress their pro-inflammatory functions (148). These macrophage responses varied depending on the strain of the parasite, suggesting it is a parasite-derived factor determining host response, supporting earlier work describing the existence of a parasite-driven virulence factor (149). These trypanosomal factors can trigger macrophages to switch toward a Th2 phenotype and include the *T. brucei* kinesin heavy chain isoform (TbKHC1) that actively induces IL-10 production and arginase activity, resulting in decreased NO production (150). When wild-type mice were infected with TbKHC1 KO trypanosomes, parasitemia was reduced and the survival of the host enhanced (150). These data suggest that the release of TbKHC1 by *T. brucei* enables the parasites to manipulate host cell metabolism by biasing the L-arginine pathway toward arginase enzyme activity, although it remains unclear whether TbKHC1 is constitutively released by viable parasites or is a byproduct from decaying or dead parasites. It has also been suggested that immunosuppression in the skin during intradermal trypanosome infection could be mediated by the combination of a mixed classical/alternative macrophage response and suppressor T cell

response (151). These findings suggest that a pro-inflammatory macrophage/Th1 response is needed for the effective control of trypanosomes in the skin, although a Th2 response seems necessary to sustain host survival in the face of a chronic infection. As such, these specific macrophage-trypanosome interactions should be investigated thoroughly for opportunities for blocking disease progression.

Dendritic Cells—Primers of Adaptive Immunity

Dendritic cells (DCs) are a group of antigen-presenting cells (APCs) that recognize and capture antigen for presentation to T cells (152, 153). Studies using i.p. *T. b. rhodesiense* infections in mice have shown that splenic DCs may be the primary APCs involved in activating VSG-specific T helper (Th) cell responses in trypanotolerant mouse lines through the upregulation of co-stimulatory receptors, the presentation of VSG peptide antigen, and the production of IL-12 (154, 155). However, subsequent VSG antigen processing and presentation was notably reduced during high parasite burdens in the mice, suggesting a trypanosome factor may be interfering with the ability of APCs to process and present antigen to T cells. Alternatively, there may be a potential impairment of the antigen presentation capacity or DC maturation during chronic infection. This remains speculative for African trypanosome infections but has been described for other infections associated with immunotolerant states (156, 157).

Skin-residing DCs include epidermal Langerhans cells and dermal DCs (158–160). Langerhans cells sample and present antigen from the epidermis to promote the adaptive immune response (161, 162). They have been shown to have a suppressive function in *Leishmania* infections by modifying the behavior of regulatory T cells (Tregs) but their role in Africa trypanosomiasis is completely unknown and remains to be established (163). Langerhans cells and dermal DCs migrate from the epidermis and dermis, respectively, to the local cutaneous draining lymph nodes to present sampled pathogen antigen to T cells (158–160, 162). Dermal DCs have also been shown to act rapidly to dermis invading protozoan parasites such as *L. major* (164). It has previously been reported in *L. major*-infected mice that epidermal Langerhans cells localized at the sand-fly bite site become activated and upregulate major histocompatibility complex molecules and co-stimulatory receptors (165). This results in cytokine release (IL-12) and the promotion of a Th1 cellular response. African trypanosomes have recently been shown to form active foci in the skin and it is likely that this would be modulated and controlled by resident DCs and Langerhans cells, similar to related trypanomastids (11, 83). Moreover, DCs might elicit a more immunotolerant state in the skin, ultimately allowing the persistence of skin foci. However, the specific roles of skin resident DCs during trypanosome infection remain to be elucidated.

T Cells—Surveying the Damage

Skin-resident T cells are another group of immune cells that survey the tissue for pathogens. The epidermal layer is patrolled by αβ effector T cells and more innate-like γδ T cells, in addition to group 2 innate lymphoid cells (ILC2s) (166–168). The γδ T cell population act with innate cells to survey tissue during the early stages of infection before the more conventional adaptive immune cells become involved. Dermal γδ T cells also express many receptors and cytokines that can alter Th1 and Th17 responses and affect extracellular pathogen, including IFN-γ, TNF, and IL-17 (169–171). ILC2s are dependent on IL-7 and constitutively secrete IL-13, thought to be central for interaction with granulocytes (167). Although T cells during skin diseases have been well-studied, particularly for psoriasis and allergies (172–174), the roles of specific γδ T cells, ILC2s, and their associated cytokines during human trypanosome infections are unclear. A study using cattle found that tsetse-transmitted *T. congolense* infection leads to the increased numbers of γδ T cells and that these cells are activated in the trypanotolerant N'Dama breed but not the more susceptible Boran breed (175). However, cattle and other ruminants possess a substantially higher proportion of γδ T cells in the peripheral blood mononuclear lymphocyte population (15–60%) than both humans and mice (<5%) (176) and their role may differ between these different hosts. Importantly, due to the induction of AAM activity observed during chronic infection, a reduction in IL-2 secretion and expression of the α-chain of the IL-2 cytokine receptor in lymph node T cells leads to inhibition of immune responses during *T. brucei* and *T. congolense* infections in mice and cattle (177–181).

While it is unclear what the potential role of skin-resident T cells may be during African trypanosome infection, insights could be gained from the pathogenesis of other parasites. For example, during infection with *Plasmodium* spp., sporozoites induce rapid immunosuppression in the skin (182–184) that affects both T and B cell functionality. *Plasmodium*-specific Tregs are also induced in the skin that expand upon re-exposure to *Plasmodium* antigens and suppress immunity to infection (185). This was found to be linked to parasite specific factors and when the sporozoite surface protein CSP was injected into the skin at low doses with CpG DNA, *Plasmodium*-specific $CD8^+$ effector T cells were significantly inhibited (182). This is hypothesized to involve regulatory B cells (Bregs) that produce immunosuppressive IL-10 and TGF-β as depletion of B cells rescued effector T cell function during malaria infection (186). Similarly, it is possible that the suppressive phenotypes observed during African trypanosomiasis also occur in the skin, hindering the successful elimination of trypanosomes. Indeed, very little inflammation or other immune responses were observed in the skin of mice with heavy burdens of skin-dwelling *T. brucei* parasites (83).

Lymphatic Invasion and Systemic Dissemination

Following skin infection, trypanosomes trigger a series of immunological events that activate resident immune cells and promote recruitment to the site of infection. This in turn delineates long-term disease outcome, specifically by determining how the parasite will disseminate systemically and establish infection. In order to achieve this, trypanosomes must

overcome the initial immunological challenge mounted in the skin and travel to the afferent lymphatics, entering the local draining lymph nodes (**Figure 1**) (11, 14–17, 151). Historical studies using dogs found that *T. brucei equiperdum* leaves the dermis via the afferent lymphatics, spreading into the draining lymph nodes before reaching the bloodstream (15). A similar sequence of events has also been shown in cattle, goats (14, 187), and recently mice (11). In this recent mouse study, trypanosomes were first detected within the local draining lymph nodes prior to their detection in the bloodstream (18 h compared to 42 h post-infection, respectively) (11). Intravital imaging of infected mice following tsetse fly bites of the skin has also been used to elucidate some of these mechanisms (17). In these experiments, a large number of parasites exhibited directional migration following trypanosome injection into the skin, traveling back and forth toward the lymphatic vessels. In addition to parasites found in the skin interstitium, parasites were also found within the afferent lymphatic vessels of the dermis. These parasites were characterized by significantly higher velocities than their extravascular counterparts, suggesting behavioral diversity. This is similar to the diverse ranges in parasite motility previously described for *T. carassii* in zebrafish (188). This would indicate that there are unknown mechanisms through which African trypanosomes are attracted toward and then invade the afferent lymphatics of the skin, allowing their subsequent systemic dissemination in the host (**Figures 1B,C**). Similar to African trypanosomes, large numbers of *Plasmodium* sporozoites have been shown to remain in the dermis while others drain to the local lymph nodes (189). These skin-resident sporozoites "glide" through the dermis and can invade the local dermal blood and lymphatic vessels before reaching the liver (190–192). Imaging studies in rodents have shown that skin sporozoites largely drain *via* the lymphatics having been phagocytosed by dendritic cells. Conversely, a minority enter directly into the bloodstream (193). Intravital imaging has shown that immunization of mice with attenuated sporozoites and *P. berghei* circumsporozoite protein inhibits sporozoite motility in the skin, resulting in inhibition of dermal blood vessel invasion (194). In addition, *Leishmania* parasites are known to form reservoirs in the skin that enhance their onward transmission to the sand fly vector (195). However, several species of *Leishmania* also disseminate systemically around the host and this has been suggested to involve infected macrophages or dendritic cells carrying the parasites to the local draining lymph nodes (94). Although African trypanosomes do not invade cells in the host, it is possible that they may be chaperoned into the local draining lymph nodes *via* macrophages or dendritic cells, either through an unknown attachment mechanism or a chemical cue.

It is also plausible that lymphatic accumulation may be driven by hydrostatic pressure, protein and/or chemical gradients, or the sensing of lymph flow (196–201). These environmental cues could direct trypanosomes toward open junctions in the lymphatic epithelium, similar to the systems used by lymphocytes for lymphatic invasion (199, 202, 203). For example, dendritic cells have been shown to respond to gradients of CCL19 and CCL21 chemokines expressed in the lymphatic vessels (204), facilitating entry into the lymphatics in the skin (205), and CXCL12 gradients have been shown to be important for the initiation of dendritic cell responses in the skin (206). However, there was no evidence for the role of several host-derived chemokines in attracting trypanosomes in a recent study (17). As African trypanosomes possess chemosensory capabilities through receptors found on the flagellum and flagellar pocket (207), it is feasible that they could respond to non-chemokine chemical gradients within the host to reach the lymphatics. For example, glucose is crucial for the metabolism of bloodstream form trypanosomes (208) and glucose concentrations in canines have been shown to be higher in the lymph than the blood (209). Glucose, lipids, or other factors essential for trypanosome metabolism, could therefore act as chemical chemoattractants for trypanosomes. The identification of potential these (tissue-specific) parasite chemoattractants merits further investigation as these might be key for understanding tissue colonization and the development of transmission-inhibiting therapeutics. Recent *in vivo* imaging has also shown that the presence of a hydrodynamic flow impacts substrate binding and swimming in trypanosomes, suggesting that the forces acting on these parasites can directly lead to changes in behavior that promote dissemination (188). Regardless of mechanisms involved, two central questions remain: (i) what are the processes deployed by trypanosomes to circumvent the immunological and physical barriers that they encounter in the skin *en route* to the lymphatic system? And (ii) are these interactions established by direct cell-cell contact between trypanosomes and host cells, or mediated by secreted factors (e.g., soluble virulence factors or extracellular vesicles)? An intriguing hypothesis is that upon differentiation, the proliferative long-slender trypomastigotes, in addition to immune cells recruited to the site of infection (e.g., neutrophils and macrophages), may actively remodel tissue architecture in a manner that facilitates movement from the site of infection and the establishment of a systemic infection. Understanding these mechanisms could again lead to tools that disrupt these behaviors, affecting transmission and the establishment of systemic infections in humans and animals.

In summary, lymphatic tropism leads to the accumulation of trypanosomes within the lymph nodes, triggering a myriad of adaptive immune responses before systemic dissemination (17, 94, 189–195). Ultimately, entry into the lymphatic system enables direct access to the bloodstream as fluids and cells drain through the thoracic duct and right lymphatic ducts re-joining the systemic circulation via the subclavian veins. Dissemination of the parasite and continued interaction with the host immune system would drive many of the consequent pathologies associated with infection. Recirculation through the dermal capillary beds also presents accessible trypanosomes to infect the tsetse fly vector, facilitating transmission and disease persistence. However, recent evidence has emerged of extravascular, skin-dwelling parasites that are also involved in transmission (11, 18, 83). Understanding this new anatomical niche has therefore become key to ongoing efforts to control the disease, particularly in the context of recently described latent HAT infections that may also be infective (210, 211).

Trypanosome Reservoir in the Skin

Historically, the presence of African trypanosomes in the host skin was a widely recognized aspect of infection that has been largely supplanted by the repeated description of *T. brucei* as a bloodstream parasite (212). Re-discovering this overlooked anatomical reservoir, and the potential implications for transmission, treatment, and control, has become a focus of trypanosome research (213). This reassessment includes the description of a metabolically unique population of *T. brucei* parasites found in the adipose tissues of various organs in the mammalian host (18). These adipose tissue form (ATF) parasites metabolize fatty acids through β-oxidation and utilize the tricarboxylic acid cycle rather than glycolysis, making them more similar to the procyclic forms found in the tsetse fly midgut rather than mammalian bloodstream forms (214). While this study did not directly find ATF trypanosomes resident in the skin, the large deposits of subcutaneous adipose (particularly in non-murine hosts) would make it an ideal environment for the parasites. Further studies have since confirmed that *T. brucei* parasites are indeed present in the skin, both interacting with adipocytes (11) and throughout the extravascular space between the panniculus carnosus and the dermis (83). However, it is currently unclear whether these skin-dwelling parasites are ATF or transcriptionally distinct.

Nevertheless, video evidence demonstrates that skin-resident African trypanosomes are motile and undergo division (83, 215). There is little overt inflammation associated with trypanosome numbers in the skin (83), although there is a rise in temperature that may serve to attract tsetse flies to sites of infection (11). Within the skin, the number of parasites cycles with the characteristic peaks and troughs associated with trypanosome infections but the cycle does not appear linked to numbers in the blood, suggesting limited transfer between compartments (83). The extent of parasite exchange between the extravascular compartment and the blood is an aspect of infection that requires further study as this has implications for treatment and pathogenesis. Skin-dwelling parasites also proceed through their life cycle and develop into characteristic "stumpy" forms that are pre-adapted to survive in the tsetse fly vector (216). The presence of stumpy trypanosomes was unequivocally demonstrated via the detection of the stumpy-specific marker PAD-1 (217) in parasites in the skin (83). Importantly, these extravascular trypanosomes contribute to tsetse fly transmission, revealing that the skin is not a "dead-end" for this supposed blood parasite. Initially, a study involving dual infection with two fluorescently tagged trypanosome strains used RT-qPCR quantification to establish that tsetse flies were predominantly infected by parasites resident in the skin rather than the blood (11). Subsequent experiments comparing infectivity with tsetse flies fed on patches of mouse skin, either with or without tissue resident trypanosomes, showed that blood and skin parasites contribute to infection, with both required for maximal transmission (83). These experiments also showed that tsetse flies could be infected by skin-resident trypanosomes in apparently aparasitemic hosts.

As HAT approaches elimination in humans, there has been an increased emphasis on understanding how the disease avoided elimination in the past. In addition to animal reservoirs (218, 219), the role of asymptomatic or latent human infections has begun to receive attention (210, 211). Counter to decades of dogma, field studies have now shown that there are individuals able to tolerate *T. b. gambiense* as latent infections without developing symptoms (211). This latent period can be extremely protracted, with a patient recently described who was infected for at least 29 years without symptoms (220). Latent individuals rarely display detectable blood parasites and are diagnosed via serology (211). However, murine experiments demonstrating that skin-dwelling trypanosomes can infect tsetse flies raise the possibility that these latent infections can contribute to transmission and act as a reservoir of infection. Recent predictive modeling also indicates that aparasitemic but infective hosts are required for a disease focus to be stable in the absence of an animal reservoir (221). This hypothesis is supported by xenodiagnosis experiments showing tsetse flies can be infected by apparently aparasitemic hosts (222, 223) and the identification of trypanosomes in historical skin samples from HAT foci (83). To date, there has been no thorough examination of trypanosomes in the skin of domestic or peri-domestic animals and this large potential reservoir requires further study to fully understand the impact on both human and animal disease.

In summary, the notion that African trypanosomiasis is a disease of just the lymphatic and circulatory systems is being re-assessed. There is strong evidence for skin-resident parasites prior to and long after systemic parasite burden, with the potential for transmission from apparently asymptomatic hosts. This has wider impacts for understanding pathogenesis, developing new therapeutics, and identifying overlooked reservoirs. These field and laboratory data also suggest that overlooked parasites in the skin of human and animal reservoirs threaten the WHO goal of eliminating HAT transmission by 2030 (218). Fully understanding the role of the skin as a biological niche for African trypanosomes will therefore likely continue to shape the field of African trypanosomiasis research.

CONCLUDING REMARKS

African trypanosomes have evolved sophisticated mechanisms to swiftly adapt to rapid changes in their microenvironment, like those encountered by metacyclic trypomastigotes delivered by the tsetse fly in the vertebrate skin when taking a blood meal. These changes are not only physical (e.g., changes in temperature and oxygen pressure) but also mechanical (e.g., transitioning from tsetse fly saliva to solid tissues such as the skin), and immunological (e.g., activation and/or recruitment of immune cells upon infection). In this scenario, it seems plausible that a combination of extrinsic factors, such as those encountered in the skin, exerts a selection pressure for trypomastigotes that are able to overcome these barriers when migrating to nearby lymphatics, leading to the establishment of systemic infections. The skin is therefore the natural point of first contact between trypanosomes and hosts, playing an active role in infection establishment and disease outcome. However, several questions remain unanswered. For example, it is unclear whether

metacyclic trypomastigotes use different environmental cues in the skin as drivers for differentiation, and if so, what the chemical nature of these differentiation signals may be. It also remains to be determined whether the initial parasite population at the site of infection remains in the skin, forming a skin-resident parasite subpopulation that is distinct from the parasites found in other tissues and organs (e.g., bloodstream forms), or whether the skin is colonized multiple times as a results of parasite migration to and from the bloodstream.

From the perspective of host-pathogen interactions, it is clear that trypanosomes release a myriad of virulence factors, including soluble products and extracellular vesicles, thought to be critical to modulate the immune response against them (224–228). In this case, it would be important to understand whether the secretion of virulence factors differs between metacyclic and long slender trypomastigotes and to what extent these secreted molecules aid in the establishment of chronic infections in the skin. Similarly, it is still unclear whether the local immune response in the skin at the site of infection helps shape the population structure of parasites that systemically disseminate within the host, and the mechanisms involved in the activation of resident skin immune cells (e.g., Langerhans cells and γδ T cells). The application of novel approaches to identify cellular heterogeneity (e.g., single cell transcriptomics and spatial transcriptomics) would help to clarify the series of events that take place in the skin before parasite dissemination; from parasite differentiation and diversity, to the activation of resident and recruited immune cells.

Finally, it is now clear that the parasites residing in the skin are central to disease transmission and present in both murine models and human clinical samples. Screening of the skin in both humans and animals is likely required to fully understand the true extent of African trypanosome infections in the field, especially as latent infections are likely still infective to tsetse flies due to skin-dwelling parasites. However, our understanding of the skin as an active immune organ delineating disease outcome in human African trypanosomiasis is in its infancy, and we anticipate that future studies should address a myriad of key basic questions in this novel field of research. As such, we need to better understand the specific mechanisms involved in establishing infection in the skin, parasitic migration from the skin, and their subsequent invasion of the lymphatics that leads to systemic infection of the host. It is also important that newly developed drugs targeting the parasite can enter the skin and remain functional, otherwise transmission will be maintained alongside increasing treatment failures as skin-dwelling parasites are selected for. In addition, it is also important to understand the host-parasite interactions that are occurring in the skin and whether they can be manipulated to act as a potential therapeutic or transmission limiting tool? How are trypanosomes reaching the lymphatics from the skin and can they be inhibited from doing so? These are all questions that need to be addressed to if we are to better understand the pathogenesis of African trypanosomiasis and develop new methods of controlling and limiting disease transmission in humans and animals.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

OA, JQ, AM, PG, RB, JB, NM, LM, and PC drafted and edited the manuscript. All authors approved the final version of the manuscript.

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REVIEW
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The Impact of Malaria Parasites on Dendritic Cell–T Cell Interaction

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Malaria is caused by apicomplexan parasites of the genus *Plasmodium*. While infection continues to pose a risk for the majority of the global population, the burden of disease mainly resides in Sub-Saharan Africa. Although immunity develops against disease, this requires years of persistent exposure and is not associated with protection against infection. Repeat infections occur due to the parasite's ability to disrupt or evade the host immune responses. However, despite many years of study, the mechanisms of this disruption remain unclear. Previous studies have demonstrated a parasite-induced failure in dendritic cell (DCs) function affecting the generation of helper T cell responses. These T cells fail to help B cell responses, reducing the production of antibodies that are necessary to control malaria infection. This review focuses on our current understanding of the effect of *Plasmodium* parasite on DC function, DC-T cell interaction, and T cell activation. A better understanding of how parasites disrupt DC-T cell interactions will lead to new targets and approaches to reinstate adaptive immune responses and enhance parasite immunity.

Keywords: dendritic cells, T cells, malaria, DC-T cell interaction, T helper cells, Tfh

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INTRODUCTION

Malaria is caused by the *Plasmodium* parasite, which affects majority of the world's population. Annually, the disease causes ∼228 million cases, resulting in 405,000 deaths. Africa accounts for about 93% of the reported cases and 94% of reported mortality cases occurring in children under the age of 5 (1). Residents in malaria endemic areas are susceptible to repeat malaria infection, with each infection resulting in modification of the hosts immune system. As well as affecting the host response to further infection (2), endemic malaria is also associated with weakened immunity to bystander infections and vaccines (3). Malaria infection has been shown to alter the phenotype and function of dendritic cells (4, 5) B cells (6, 7) and T cells (7–10) causing a disruption in the host immune response.

PLASMODIUM LIFE CYCLE

Plasmodium has a complex life cycle that occurs in two hosts; the female *Anopheles* mosquito (sexual reproductive stage) and a vertebrate host (asexual development stage). The latter begins when an infectious female *Anopheles* mosquito probes the dermis of a mammalian host as it takes a blood meal, releasing a highly motile form of the parasite, sporozoites,

from its saliva (**Figure 1A**) (11, 12). Not all sporozoites manage to reach the blood vessel and those that remain in the dermis are either destroyed or drained into the lymphatics where the host's immune system eliminates them (13, 14). Those that manage to enter the bloodstream circulate and enter the liver through a process known as traversal, to gain access to a suitable hepatocyte (15, 16). Once inside a suitable hepatocyte, the sporozoite forms a parasitophorous vacuole (PV) and undergoes pre-erythrocytic schizogony, forming merozoites that accumulate within the parasitophorous vacuole and bud off the hepatocyte in structures called merosomes, clearing the liver of parasites (**Figure 1B**). The merosomes enter the bloodstream, releasing the encapsulated merozoites to infect red blood cells (RBCs) (17–19).

In the blood, the free merozoites attach to, and subsequently invade the RBC, initiating the erythrocytic stage of the parasite life cycle. Once inside the RBC, the merozoite matures in three morphologically distinct stages, namely the ring, trophozoite, and schizont stages. During the maturation stages the RBC undergoes a number of structural and functional changes that alter the architecture of the RBC membrane (**Figure 1C**) (20). Key amongst the structural changes is the expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), a vital parasite protein that is central to *P. falciparum* pathogenesis (21–23). PfEMP1 is expressed on the surface of parasite infected RBCs (iRBC) and enables iRBCs to sequester and cytoadhere to vascular endothelium, preventing their destruction in the spleen. Apart from the structural changes that occur to the RBC, the parasite also undergoes nuclear division producing merozoites that fill the PV (the schizont stage). The merozoites egress from the iRBC and invade other RBCs initiating another cycle for parasite replication.

After rounds of schizogony, some *P. falciparum* trophozoites commit to sexual development and form gametocytes. The gametocytes undergo five stages of maturation while being sequestered in the bone marrow. Only stage five gametocytes re-enter circulation and are taken up by a mosquito during a blood meal (24).

Interaction between DCs and *Plasmodium* parasite occurs at various points during the life cycle of the parasite in a human host (**Figure 1**). The parasite encounters DCs in the skin (**Figure 1A**) (13, 25), the liver (**Figure 1B**) (26, 27), and the blood and spleen (**Figure 1C**) (4). Tissue resident DCs in each of the sites can phagocytose parasite components and initiate specific immune responses to the parasite.

DENDRITIC CELLS

DCs are mononuclear phagocytic cells that are found in the blood, lymphoid organs and all tissues. They are the most effective professional antigen presenting cells in the body due to their ability to capture, process and present antigen on either major histocompatibility complex (MHC) class I or MCH class II molecules and activate naive CD8 or CD4 T cells (28, 29). DCs are central in initiating and regulating adaptive immune responses and act as a bridge between the innate and adaptive arms of the immune system. DCs differentiate from hematopoietic stem cells (HSC) (30) in the bone marrow to immature DCs, which circulate in blood and home to various peripheral tissues. Immature DCs recognize a range of danger signals such as pathogen-associated molecular patterns (PAMPS) which are found on pathogens and damage associated molecular patterns (DAMPS) which are released by injured host cells (31), through a number of pathogen recognition receptors (PRRs) (32, 33). Ligation of PRRs initiates DC phagocytosis, resulting in ingestion of the invading pathogen and initiation of DC maturation and migration into the lymph node where they present antigens to naive T cells (34). The maturation process results in increased expression of MHC surface molecule coupled with pathogen antigens and costimulatory molecules (CD80, CD86, and CD40), which are key in proliferation and differentiation of naive T cells into effector cells (35). DCs also secrete cytokines and chemokine that attract other immune cells to sights of infection/injury and influence the outcome of T and B cells responses (36).

DCs are lineage negative cells [that is they are defined by the exclusion of T cells (CD3), B Cells (CD19, CD20) natural killer cells (CD56), monocytes (CD14, CD16) and progenitor cells (CD34)] and express MHC class II (HLA-DR) and are broadly classified into either plasmacytoid DCs (pDCs) or conventional DCs (cDCs). In humans, pDCs are characterized by expression of CD123, CD303 (BDCA-2) and CD304 (37) and are known to produce large amounts of type I interferon in response to viruses (38). This is enabled by the high expression levels of toll-like receptor 7 (TLR7) and TLR9, which recognize nucleic acids from viruses, bacteria, and dead cells (39, 40). cDCs specialize in priming and presenting antigen to T cells. They can be further classified into cDC1 and cDC2. cDC1 express BDCA-3/CD141, CLEC9A, and XCR1 and have enhanced ability to cross present antigen (41) to CD8 T cells. cDC2 express BDCA-1/CD1c and have a wide variety of pattern recognition receptors (PRR's) and a good capacity to stimulate naive CD4 T cells but they have a poor ability to cross-present antigens to CD8 T cells compared with cDC1 (37, 40).

DCs are central in any immune response as they sense pathogens and initiate immune responses and are present at various sites during the life cycle of the *Plasmodium* parasite. As discussed later, the parasite's numerous immune evasion mechanisms interfere with DC function, thus altering downstream immune effector functions and the course of the disease.

T CELLS

T cells develop in the thymus from the common lymphoid progenitors which originate from bone marrow derived hematopoietic stem cells (42). After development and maturation, naiveT cells exit the thymus and enter circulation expressing either CD4 or CD8 and an antigen-recognizing T cell receptor (TCR) on their surface. The naive T cells home to secondary lymphoid organs (SLO) where they await a signal from DCs to become activated.

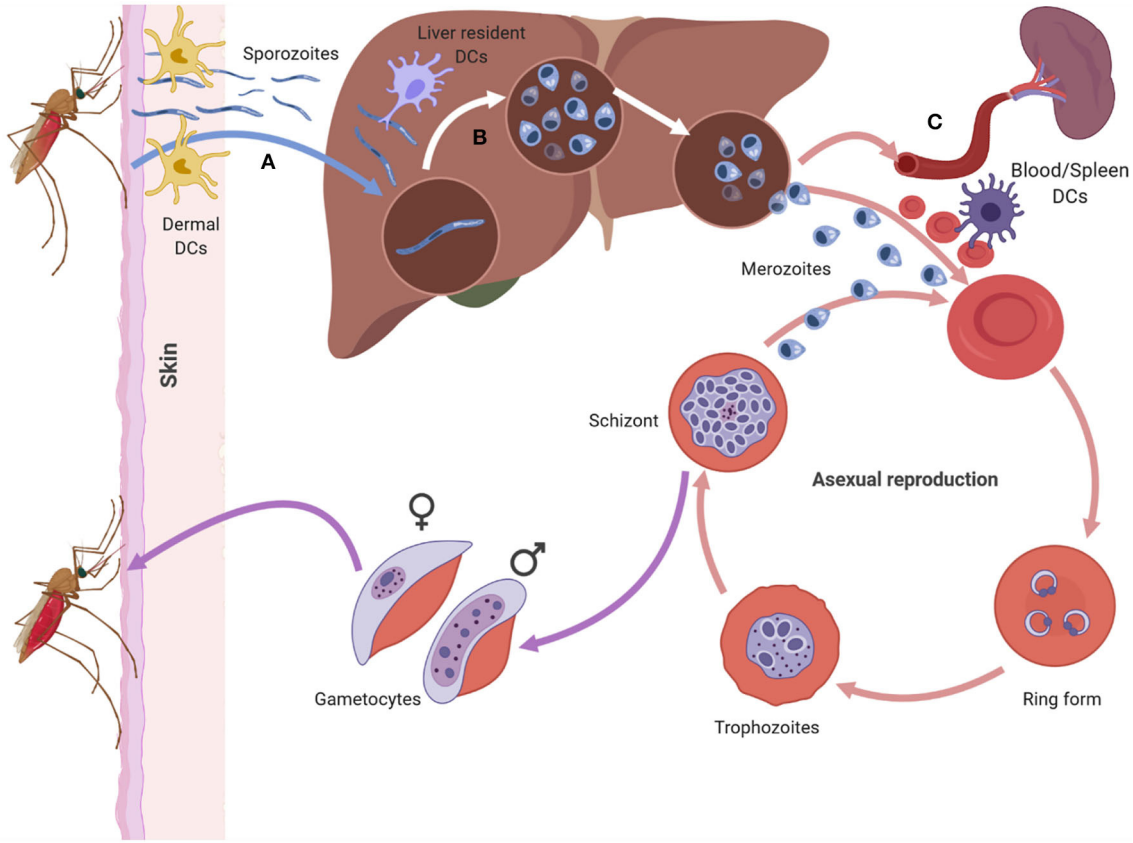

FIGURE 1 | The asexual life cycle of Plasmodium parasite begins when an infected mosquito injects highly motile sporozoites into the skin of the host. The sporozorites enters the bloodstream and migrates to the liver, where it traverses multiple hepatocytes before infecting one. Inside the hepatocyte the sporozoite undergoes pre-erythrocytic schizogony forming merozoites that accumulate and bud off the hepatocyte in structures called merosomes. Merosomes enter the bloodstream and release merozoites which invade RBC, initiating the erythrocytic stage of asexual development. At this stage the parasite develops inside the RBC in distinct forms namely the ring, trophozoite, and schizont form. The schizont, lyses releasing merozoites into the blood stream which reinvade RBCs starting a fresh round of asexual development. After rounds of erythrocytic schizogony some of the asexual parasites develop into gametocytes and are taken up by a mosquito during a blood meal. Dendritic cells can interact with sporozoites in the dermis **(A)**, the liver **(B)** and the blood and spleen **(C)**. The DCs at each site encounter the parasite in its different forms (Figure was created using BioRender).

CD8 T CELLS

Naive CD8 T Cells are activated by recognition of foreign or neoantigens presented by MHC class I molecules on DCs in the secondary lymphoid organs. Additional co-stimulatory signals and cytokines from DCs and/or CD4 T cells help in differentiation and clonal expansion of the T cells (43–46). The activated effector CD8 T cells migrate from the secondary lymphoid organs into circulation and identify their target cells which express cognate antigens on the cell surface bound to MHC class I. MHC class I is expressed on all nucleated cells except red blood cells. The target cells are killed by effector CD8 T cells through cell contact dependent cytolysis by releasing granzyme B and perforin (47–49). Perforin creates pores on the plasma membrane of the target cell; the pores allow granzyme B to enter the target cell and initiate apoptosis resulting in killing of infected cells. After clearing the invading pathogen, antigen specific effector CD8 T cells die off and a small number differentiate into memory CD8 T cells (45, 50).

Antigen specific CD8 T cells have been observed in the peripheral blood of residents from a malaria endemic area (51) and after vaccination of malaria naive individuals with irradiated sporozoites (52). In experimental mouse models of malaria, CD8 T cells specific for sporozoites antigens, liver stage antigens, and blood stage antigens were observed when mice were challenged with radiation attenuated sporozoites (53). It is believed that the priming of CD8 T cells against the pre-erythrocytic stages of *Plasmodium* occurs in the skin draining lymph nodes when sporozoites are injected into the skin by an infected mosquito (14, 54). These CD8 may offer protection against subsequent *Plasmodium* infections as incubation time in the liver offers a short window of opportunity for the CD8 T to mount an effective response.

CD4 T CELLS

CD4 T cells, on the other hand, recognize antigens presented by MHC class II molecules, which are present on antigen presenting cells such as B cells, macrophages, and dendritic cells. CD4 T cells generally provide help to B cells in the germinal center enabling class switching and production of high-affinity antibodies (55). They also aid in CD8 T cell activation by licensing DCs (56–58) or directly signaling CD8 T cells via CD40 (59). They also secrete cytokines such interferon gamma (IFNγ), C-X-C motif ligand 9 (CXCL9), CXCL10 (60) interleukin-2 (IL-2) (61–63), and IL-21 (64) that are key in shaping immune responses. The diverse range of CD4 T cell functions are handled by distinct subsets of cells. The cytokine milieu in the microenvironment during CD4 T cell activation dictates the specific cytokine signaling networks and transcription factor activated for the differentiation of naive CD4 T cells into T cell subsets. The cytokines involved in CD4 T cell differentiation are produced by DCs and other innate immune cells, driving the cells to differentiate into either T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), follicular helper T cell (Tfh), induced T-regulatory (iTreg), or the regulatory type 1 cells (Tr1).

Tfh cells have been a recent focus of interest in malaria immunology. Tfh cells express C-X-C motif receptor 5 (CXCR5) on their surface and are vital in the development of humoral immunity (55). Differentiation of CD4 T cells to Tfh is a multistep step process that first begins with DC interacting with a naive CD4 T cells in the T cells zone (**Figure 3**). This interaction results in the formation of pre-Tfh cells expressing CXCR5 that migrate to the T-B cell border of the SLO (65). At the T-B cell border and interfollicular zone, pre-Tfh interact with antigen specific B cells to initiate the B cell dependent phase of Tfh differentiation, which is characterized by upregulation of transcription factor B cell lymphoma 6 (Bcl-6) (66) and commits the Tfh lineage. After events at the T-B cell border, the Tfh migrates into the follicle and interacts with B cells forming germinal centers, where B cells undergo affinity maturation and heavy chain class switching, resulting in the production of high-affinity antibodies with enhanced effector functions (67). Tfh differentiation involves a number of cytokines such as IL-6, IL-21 (68), IL-12 (69), IL-27 (70), and TGF-β (71). These cytokines initiate signal transducer and activator of transcription 1 (STAT1), STAT3 (72) and STAT4 (73). The STATs upregulate the transcription factor B cell lymphoma 6 (Bcl-6), the master transcription factor in Tfh differentiation. Apart from cytokines, other signals required during differentiation of Tfh cells include the inducible costimulator (ICOS)- inducible costimulatory ligand (ICOSL) signaling (74, 75) and CD40-CD40L signaling.

CD4 Tfh cells are essential for promoting antibody response that aid in resolving malaria infection (76, 77). In malaria infected humans and mice, Tfh cells adopt a Th1 like phenotype that expresses Tbet+ PD-1+, CXCR5+, CXCR3+, and secretes IFNγ (77, 78). This Tfh phenotype does not provide adequate help to B cells resulting in suboptimal antibody responses. Dysfunctional DCs that are induced by malaria may play a role in initiating this Th1-like phenotype that skews humoral response (**Figures 2D,E**).

THE IMPACT OF *PLASMODIUM* ON DC-T CELL INTERACTIONS

Activation of T cells requires interaction with DCs, which provide three key signals (**Figure 2**). Signal 1 occurs when T cells recognize cognate peptide antigen presented on either MHC I or MHC II on the surface of DCs via their T cell receptor (TCR). MHC-TCR interactions trigger activation of the T cells and initiates downwards signaling through immunoreceptor tyrosine-based activation motifs (ITAMs) (79). Besides TCR-antigen-MHC complex, a second signal, the costimulatory signal, is required to initiate and sustain T cell activation and proliferation. Co-inhibitory molecules (immune checkpoints) also form part of the second signal, but they downregulate immune responses (**Figure 2B**) (80, 81). Key costimulatory molecules involved in T cell activation include CD28 (binds to CD80/86 on DCs), ICOS (binds to ICOSL on DC), OX40 (binds to OX40L on DCs), and CD40L (binds to CD40 on DCs), are key in T cell activation, differentiation and survival (**Figure 2A**). These costimulatory signals work in synergy with the TCR-antigen-MHC complex to enhance the activation of T cells. Co-inhibitory molecules such as cytotoxic T-lymphocyte-associated protein 4 [(CTLA-4), competes for binding to CD80/86 with CD28 on DC], and programmed cell death-1 [(PD-1), binds to PD-1L] work to suppress the activation signal from TCR-antigen-MHC complex (**Figure 2B**). Once the T cell has received TCR-antigen-MHC complex signaling together with adequate co-stimulation, it receives a third signal in the form of cytokines that are secreted by DCs. As mentioned above, cytokines are important in deciding the fate of CD4 T cell differentiation toward a particular subset. Subsets of CD4 T cells include Th1 type (CD4 T cells exposed to the cytokine IL-12), Th2 (IL-4), Th-17 (IL-6, IL-23), Tfh (IL6, IL21), and iTreg (TGF-β) (**Figure 2**).

Tfh differentiation is a multistep process that requires signal 1 in the form of antigen presented on MHC II by DCs (**Figure 3**). This interaction occurs at the T cell zone and involves the costimulatory molecules CD80, CD86, and inducible costimulatory ligand (ICOSL) on DC that interact with CD28 and ICOS to generate signal 2 in T cells. The CD28-CD80/86 interaction results in the upregulation of ICOS on T cells that interacts with ICOSL on DCs. The cytokine (signal 3) produced by DCs that helps in the initial process of Tfh differentiation is IL-12 (82). A combination of CD28-mediated signaling on T cells and IL-12 is adequate to upregulate the expression of Bcl-6, IL-12 also induces IL-21 production in T cells, which acts in an autocrine manner to ensure growth and survival of pre-Tfh. Bcl6 expression upregulates CXCR5 expression allowing the pre-Tfh cells to migrate to the T cell-B cell zone (83). At this zone, the Tfh cell interacts with B via ICOS-ICOSL committing the cell to the Tfh lineage and further upregulating CXCR5 and SAP (67). The CXCR5 and SAP expressing Tfh cells then move into the B cell follicle and form stable, long-lasting interactions with B cells forming germinal center where Tfh cells aid in class switching and generation of long-lived plasma cells that secrete high-affinity antibodies. Germinal center Tfh cells are also involved in the formation of long-lived plasma cells and memory B cells (84, 85).

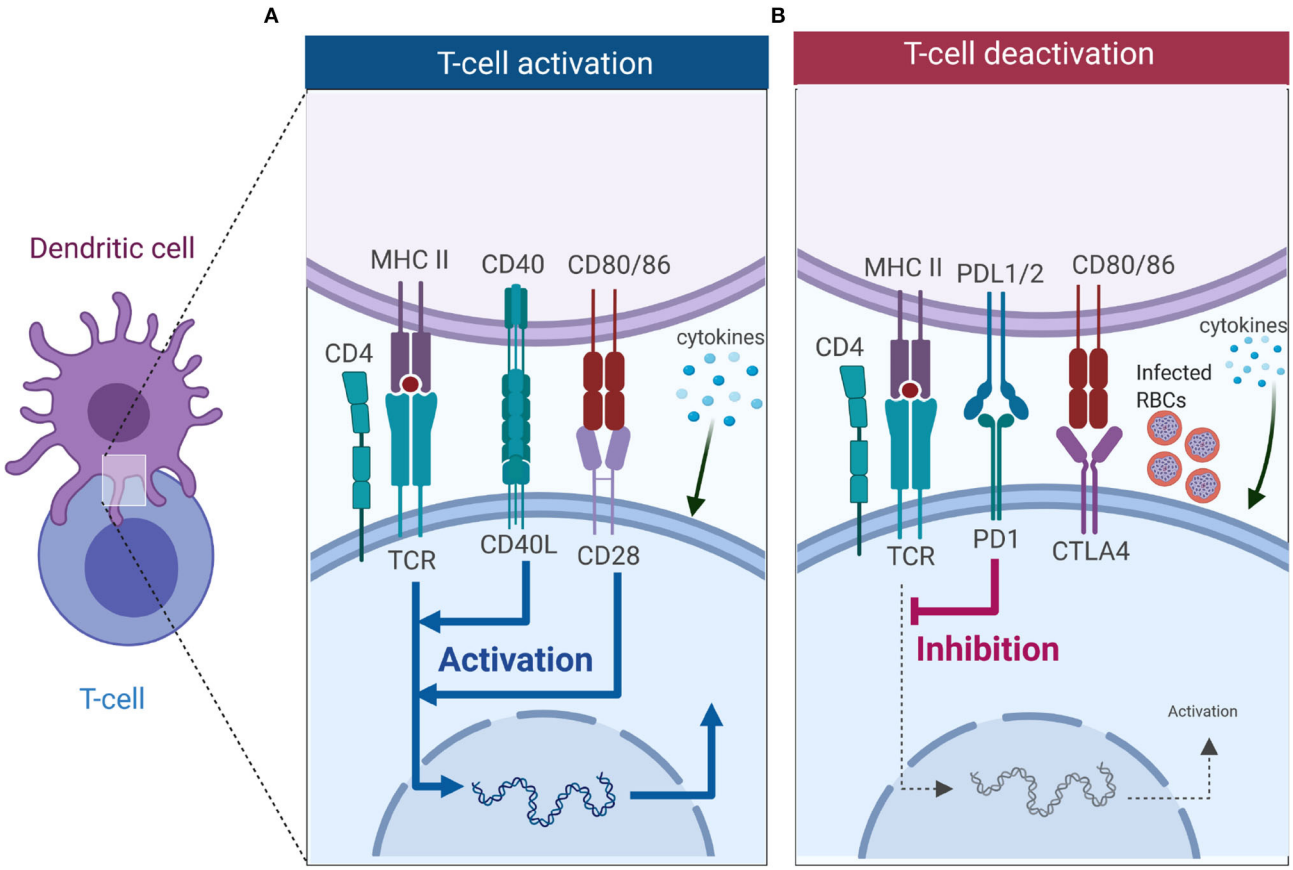

FIGURE 2 | T cell activation or deactivation requires three signals from DCs. **(A)** T cell activation requires signal 1 in the form of TCR interacting with antigen-MHC complex which is key in innating downward signaling through ITAMs. Interaction of co-stimulatory molecules (interaction of CD40-CD40L, and CD80/86-CD28) form part of signal 2 as they work in tandem with TCR-antigen-MHC complex to enhance TCR signaling and initiate T cell proliferation. Signal 3 comes from DCs in the form of cytokines, and in CD4 T cells it is key in dictating which subset it will differentiate into. **(B)** Co-inhibitory molecules also form part of the second signal but unlike co-stimulatory molecules they inhibit TCR signaling, thus dampening immune responses. Interaction of PD-1 with PD-L1 inhibits T cell activation, while CTLA4 competes for binding with CD28 to CD80/86 and successful binding of CTLA4 to CD80/86 nullifies CD28-CD80/86 activation signal. Malaria has been shown to induce expression of PD-1, LAG3 and CTLA-4 on CD4 T cells, and this inhibits the activation signal from DCs (Figure was created using BioRender).

P. FALCIPARUM IMMUNE EVASION AND SUPPRESSION OF IMMUNITY

P. falciparum is equipped with multiple mechanisms which it uses to evade the host's immune system. These mechanisms include antigenic variation of surface antigens (VSA) expressed on iRBCs such as PfEMP1 which is encoded by the *var* genes (21), sub-telomeric variable open reading frame (STEVOR) encoded by the *stevor* genes (86, 87) and repetitive interspersed repeats (RIFIN) encoded by the *rif* genes (88, 89). Antigenic variation of VSAs normally occurs when the parasite is under intense immune pressure from the host in order to avoid recognition by various immune cells (90, 91). The expression of different VSAs on iRBCs allows the parasite to establish new infections (92). VSAs are key in sequestration and cytoadherence of maturing parasites (trophozoite and schizonts) and rosetting (93, 94). Merozoite surface protein (MSP) polymorphism (95–97) and complement evasion by surface proteins PfMSP3.1 (98), Pf92 (99), and PfGAP50 (100) expressed on merozoites and gametes are other mechanisms used by the parasite to escape elimination by the immune system.

Apart from immune evasion, ongoing *Plasmodium* infections have been shown to reduce immunogenicity of vaccines in children. Antibody responses to *Salmonella typhi* and tetanus vaccines were greatly reduced in malaria infected children compared to healthy control and children with other acute illnesses (3). Adults with previous exposure to *P. falciparum*, showed no response to malaria antigen, regardless of disease severity, and reduced response to non-specific antigens (2). Infection of influenza-immune mice with *P. chabaudi* resulted in a decrease in influenza specific antibodies and plasma cells resulting in a loss of protective immunity against influenza (101), which recovered several weeks after parasite clearance. This indicates that malaria infections somehow suppress immune function by interfering with the development of adaptive immunity. Ongoing malaria infection reduces immunogenicity to heterologous vaccines and malaria derived antigens. The

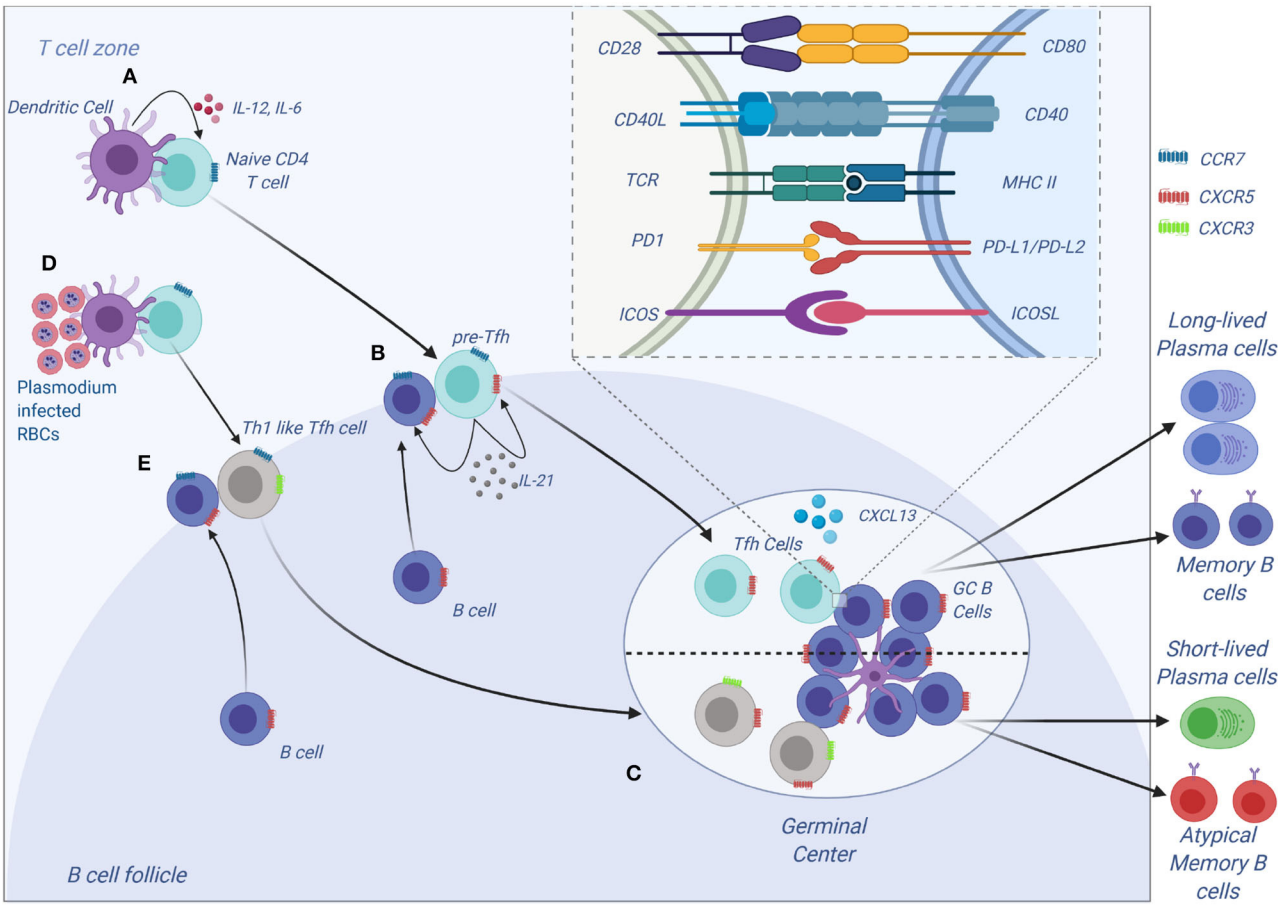

FIGURE 3 | Tfh development and function. **(A)** Tfh differentiation begins when an activated DC primes a naïve CD4 T cells. Interaction between ICOS on CD4 and ICOS-L on DCs results in upregulation of Bcl-6 and CXCR5 on CD4 T cells. IL-12 and IL-6, help maintain expression of Bcl-6, and IL-12 induces upregulation of IL-21 which is essential for survival of the pre-Tfh cell. Initial CD4 T cell activation also results in upregulation of PD-1. The Bcl-6+CXCR5+ PD-1+ pre–Tfh cell then migrates to the T cell–B cell border. At the same time, antigen-activated B cells upregulate CCR7 and migrates from the B cell follicle to the T cell–B cell border. **(B)** At the T cell–B cell border, the pre-Tfh cell engages with the B cell. Interaction between antigen-MHC on B cells with TCR on pre-Tfh, and ICOSL (B cell) with ICOS (pre-Tfh cell), fully commits the cell to Tfh lineage. This results in upregulation and maintenance of Bcl-6, SAP, and CXCR5, while downregulating CCR7 on T cells. **(C)** The Tfh and B cells move deeper into the B cell follicle forming GCs. Tfh cells in the GCs promotes B-cell maturation, class switching and affinity maturation via the cytokines, IL-21 and IL-4, and the molecules, CD40L and PD-1. Both Tfh and GC B-cell are necessary for generation of B-cell memory and long-lived plasma cells. **(D,E)** *Plasmodium*-induces polarization of T follicular helper (Tfh) cells to Th1 like phenotype that expresses Tbet PD-1, CXCR5, CXCR3 and contributes to the inefficient acquisition of humoral immunity to malaria. Malaria infection in mice and humans induces secretion of Th1-polarizing cytokine that drive the activation of Th1-like Tfh cells that exhibit impaired B cell helper function, thus contributing to germinal center dysfunction and suboptimal antibody responses (Figure was created using BioRender).

exact mechanism used to induce this suppression is yet to be uncovered.

The suppression of immune function seen in malaria infection could be attributed to DC/iRBC interaction which alter the maturation state and function of DC in both humans (4, 102, 103) and mice (8, 104). DCs exposed to iRBC *in vitro* and *in vivo* have reduced expression of MHC on the surface and are unable to form stable interactions with CD4 helper T cells (104). The DCs also downregulate key costimulatory molecules, such as CD86, CD80, CD40, and secrete IL-10 (105), providing a suppressive environment for CD4 T cell development. This hampers their ability to activate naive CD4 T cells and a failure to generate Tfh cells that are critical in the formation of germinal center and generation of protective antibodies against malaria infection (77, 104). In contrast, other *in vitro* studies have shown that DCs exposed to iRBC successfully activate T cells, but induce their polarization toward a Th1 phenotype that inhibits commitment to Tfh cell linage, thus affecting humoral responses (103, 106).

WHAT HAPPENS TO DCS DURING A *PLASMODIUM* INFECTION?

During the *Plasmodium* parasite life cycle, different forms of the parasite interact with resident DCs in various organs as it establishes infection. Sporozoites from infectious mosquitoes that are injected into the dermis interact with resident DCs in the skin (107). The sporozoites reach the liver interact with

Kupffer cells, hepatocyte, and liver sinusoidal endothelial cells and resident DCs in the liver (108). The blood stage of the parasite interacts with DCs in the blood (4) and spleen (109–111).

DC INTERACTION WITH *PLASMODIUM* SPOROZOITES IN THE SKIN AND LIVER

The skin is the first point sporozoites encounter DC as they are inoculated by infected mosquitoes (**Figure 1A**). Studies conducted in mice have shown that only a small percentage of inoculated sporozoites leave the site of injection as most end up trapped in the dermis or enter the lymphatic system rather than the blood vessel (13). Other sporozoites infect keratinocytes, hair follicles, and develop into exoerythrocytic forms of the parasite (112). Sporozoites that are trapped in the dermis are phagocytosed by resident DCs which migrate to the skin-draining lymph node and can prime CD4 (113, 114) and CD8 (14) T cell responses. The immune response toward the sporozoite stage of the parasite may protect against subsequent challenges from infected mosquitoes (113).

The sporozoites that mange to enter blood circulation move to the liver and must traverse the sinusoidal barrier to access hepatocytes (**Figure 1B**) (115). The liver environment is tolerogenic due to the presence of IL-10 and TGF-β which are secreted by Kupffer cells (KC) and liver sinusoidal endothelial cells (LSEC) (116). These cytokines reduce expression levels of MHC class II and costimulatory molecules on the surface of liver resident DCs compared to resident DCs in lymphoid organs and those circulating in the blood (117) thus reducing their capability to activate T cells (118, 119). The tolerogenic environment of the liver could play a role in sporozoite immune evasion as DCs and other immune cells in the liver act to suppress adaptive immune responses which would lead to the elimination of sporozoites (116, 120).

Apart from DCs, the liver has other potential APCs that can present antigens to the adaptive immune system; this includes Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), and hepatocytes. LSECs are scavenger cells that express MHC class I and II molecules, low levels of CD86, and the adhesion molecules ICAM-1, VCAM-1, and dendritic cell specific intercellular adhesion molecule3-grabbing non-integrin (DC-SIGN). In mice, these cells have the ability to cross present antigens in the liver and activate CD8 T cells, but the T cells are generally tolerized due to the secretion of IL10 and PGE2 by LSECs (121, 122). KCs are resident tissue macrophages found in the liver that express MHC class I and class II molecules, ICAM-1, CD86, CD80, and can activate naive CD4 and CD8 T cells *in vitro* (123, 124). The role of KC as an APC is controversial as *in vitro* experiments show that they inhibit T cell activation by secreting IL-10 (125), but activation of KCs via TLR3 increased the expression of MHC class II and their APC function (126). Kuniyasu et al. (127) showed that the liver had the ability to retain adoptively transferred T cells. The T cells proliferated and expanded in the liver, but the expansion was followed by apoptosis, which was initiated by KCs (127). It was later shown that KCs induce T cell apoptosis via the FAS-FAS-L signaling pathway (128).

Hepatocytes express MHC class I and ICAM-1 in their steady state and during inflammation they have been shown to express MHC class II CD40L, CD80 and CD86 and are capable of activating CD8 T cells (129). Their role in generation of malaria liver immunity has been controversial with different studies using mouse models drawing different conclusions of their role in the generation of pre-erythrocytic immunity. Intrasplenic injection of parasite infected hepatocytes in mice resulted in T cell mediated immunity against *P. yoelii* and *P. berghei* infections (26), thus showing that hepatocytes are capable of activating T cells. Another study demonstrated that parasite infected hepatocytes undergo apoptosis, thus providing liver DCs with a source of *Plasmodium* antigens for initiating the adaptive immune response (130). This idea has been challenged and it has been suggested that DCs could obtain *Plasmodium* antigens directly from viable infected hepatocytes. This is supported by the fact that DCs have the ability to acquire antigens from other live cells and cross present to CD8 T cells (131).

Chakravarty et al. (14) showed that cross presentation of *Plasmodium* antigens by DCs was key in CD8 T cells activation and this occurred in the skin draining lymph node, not in the liver, and the activated T cells recirculated to the liver (14). Indicating that DCs in the skin that encounter sporozoite play a crucial role in generating T cell mediated liver immunity. Recently Kurup et al. (27) showed that during a malaria infection, a subset of monocyte derived CD11c+ APC infiltrate the liver after hepatocyte infection by *Plasmodium* parasite and acquire *Plasmodium* antigens. The monocyte derived CD11c+ APC present the antigens to naive CD8 T cells in the liver draining lymph node, priming them and initiating T cell mediated immunity against *Plasmodium* infection (27).

While there are still some gaps into how the generation of liver immunity against *Plasmodium* infection is acquired, it is clear that APCs, especially DCs, play a central role. Hepatocytes may play a part in the generation of liver immunity by providing parasite antigens to DCs but the exact mechanism of this is yet to be uncovered. A better understanding of DC and hepatocyte involvement in the generation of liver immunity is required and also the roles played by KCs and LSECs. The use of humanized mice might provide an opportunity to further investigate skin and liver immunity against *P. falciparum* (132, 133).

DC INTERACTION WITH *PLASMODIUM* DURING THE BLOOD STAGE OF MALARIA

The blood stage of the malaria parasite life cycle provides several opportunities for DC in the blood and spleen to interact with infected RBC (**Figure 1C**). This stage requires remodeling of the RBC to enable the parasite to survive (134) and results in the expression of parasite antigens on the RBC surface. These antigens, in particular PfEMP, play a key role in immune evasion and vascular sequestration/cytoadherence to avoid splenic clearance (21, 135). It has been suggested that PfEMP1 may be involved in modulation of DC function via interaction with CD36 (4, 136).

Maturation of iRBCs (schizont stage) results in lysis of the iRBCs, releasing merozoites into circulation and the contents of the PV such as the parasites digestive vacuole which contains hemozoin and waste products. The free merozoites have a short window to invade new RBCs (137) and those that fail to invade remain in circulation where they are phagocytosed by immune cells or cleared in the spleen. Parasite waste products and hemozoin do interact with DCs but their overall effect on DC function is contradictory. The effect of hemozoin on DCs has yielded varying results with some studies showing that hemozoin is capable of activating DCs (138) while others showed that DC maturation and function was inhibited by hemozoin (8, 139). The varying results could be due to the different methods that were used to generate hemozoin with contamination by parasite DNA being a potential confounding factor (140).

Overall, the blood stage has an abundance of parasite antigens that DCs can use to mount an immune response. However various immune evasion mechanisms, such as antigenic variation of VSAs (141, 142) and sequestration of mature schizont and trophozoites in blood capillaries (143) thus avoiding splenic clearance (144, 145) the slow acquisition of immunity. DCs at this stage are critical in maintaining an immunological balance between parasite burden and a sufficient immune response. Immune evasion by the parasite could cause an increase in parasite burden resulting in severe pathology, while an excessive and uncontrolled immune response may lead to the development of a severe life threating cerebral malaria (146–148).

Studies of blood stage infections with DC have largely employed DCs prepared from peripheral blood monocytes or isolated from peripheral blood of uninfected individuals (4, 102). Fewer studies have analyzed the phenotype and function of peripheral blood DCs from individuals who are currently undergoing a malaria episode (103, 149, 150). In this context, *in vivo* mouse models of malaria have been particularly helpful to understand the tissue responses of DC, for example splenic DC and allow temporal analysis of how *Plasmodium* infection changes DC phenotype (8, 104).

IN VITRO DC INTERACTION WITH PLASMODIUM

In vitro studies have been used to identify the mechanisms used by the parasite to modulate DC function. These studies have either used human monocyte derived dendritic cells (moDCs) or bona fide DCs to assess DC- *P. falciparum* interactions.

Urban et al. (4) showed that when moDCs were co-cultured with *P. falciparum* iRBC, at a ratio of 1:100, and later stimulated with lipopolysaccharide (LPS), exhibited a decreased expression of key maturation markers (CD40, CD80, CD86, and CD83) (4). Once moDCs were exposed to iRBC, they lacked the capacity to activate allogeneic T cells (4). This modulation of DC maturation may result from an interaction between CD36 on DCs with PfEMP-1 on iRBC (105). A subsequent study found that a ratio of 1 DC: 100 iRBC inhibited LPS induced moDCs activation, cytokine production, and allogeneic T cell activation regardless of CD36-binding with iRBC (102). The high ratio of DC to iRBC coincided with an increase in apoptotic and necrotic cells, which was observed in both PfEMP1-deficient iRBCs and PfEMP1 expressing iRBCs, this could account for the failure of DCs to respond (102). At low iRBC to moDC ratio (10:1), moDCs made a modest response to LPS induced maturation and retained their ability to secrete cytokines and activate T cells (102). Elliot et al. (102) were unable to point out the mechanism used by *P. falciparum* to modulate moDC function, although they found that hemozoin, from iRBC lysate, did not inhibit LPS maturation of moDCs (102). The studies show that a dose-dependant relationship exists between iRBC and moDCs inhibition and dose range experiments are an essential part of ensuring experimental reproducibility in the future.

Another study found that at a low ratio of 10 iRBC per moDCs did not trigger the upregulation of HLA-DR, CD83, or CCR7 on moDCS (151), contradicting the study by Elliot et al. (102). At a ratio of 100 iRBCS per moDCs, moDCs were able to secrete IL-1β, IL-6, IL-10, TNF-α, and upregulate the chemokine receptor CXCR4 (151). Exposure of moDCs to schizont lysate resulted in an increase in the expression levels of CD86 while CD80 and HLA-DR levels remained unaffected even at high concentration of schizont extract (106). Exposure to schizont lysate, followed by LPS stimulation, did not affect the maturation of moDCs. The schizont lysate exposed moDCs maintained their ability to differentiate allogeneic T cells into Th1 and regulatory T cells (Treg) that secrete large amounts of IFN-γ. Additionally, the generated Tregs also secreted IL-10 and TGF-β (106).

The different *in vitro* studies looking at the effect of *P. falciparum* on moDCs have yielded varying results. This could be attributed to the use of the *Plasmodium* parasite at different stages of development in the RBC. Another explanation could be that the studies used different experimental methods in the isolation of the *Plasmodium* infected red blood cells and in the generation of moDCs.

Few studies have examined the effect of *P. falciparum* on cDCs and pDCs due to their low numbers in peripheral blood. One study examined the effect of *P. falciparum* on cDC2 and pDCs (103). The co-culture of cDC2 with *P. falciparum* at a ratio of 1:3 resulted in the upregulation of maturation markers (CD80, CD86, CD40, and HLA-DR) and inflammatory chemokines CCL2, CXCL9 and CXCL10 but did not induce secretion of inflammatory cytokines. Exposure of cDC2 to iRBC did not inhibit cytokine secretion in response to LPS, which was contrary to what was observed with moDCS (103). The low ratio of iRBC to DC may account for this observation as the study did not use a higher ratio of iRBC to cDC2. The cDC2 exposed to iRBC maintained their ability to present antigens and activate naive T cells to polarize them toward a Tfh1 phenotype that secretes IFN-γ (103). The study also found that crosstalk between pDCs and cDC2 was important in shaping immune responses against malaria. The co-culture of pDCs and cDCs resulted in the upregulation of HLA-DR, CD86, and CD40 on pDCs and CD80 and CD86 on cDC2. There was also an increase in the secretion of interferon alpha (IFN-α) by pDCs and chemokines CXCL9 and CXCL10 by cDC2. This cross-talk between these two DCs was contact dependent, suggesting cell to cell interaction is necessary to initiate chemokine secretion (103). The study highlighted the

importance of cell to cell interaction which is crucial in trying to understand immune responses in malaria.

In mouse studies using bone marrow derived dendritic cells (BMDCs), *P. chabaudi* schizonts were shown to be able to activate BMDCs to produce the pro-inflammatory cytokines IL-12 and TNF-α. The *P. chabaudi* exposed DCs did not inhibit LPS activation, contrary to what was observed with *P. falciparum* exposed human DCs (152).

EX VIVO DC INTERACTION WITH *PLASMODIUM*

A number of studies have compared peripheral blood DCs in varying malaria transmission settings and different at-risk groups. In Kenya, children hospitalized with either mild or severe malaria were found to have a lower number of DC expressing HLA-DR and a lower number of circulating DCs compared with healthy children (149). A follow up study revealed that the expression levels of HLA-DR was reduced on monocytes and cDC but not on pDC and that DC modulation continued during convalescence. An increase in the frequency of BDCA3+ cDC1 in the peripheral circulation was also observed during the course of the malaria infection (153).

A similar study was conducted in Mali looking at the function of DCs in children with severe malaria from the Dogon and Fulani community. The two communities reside in the same geographical region and are exposed to the same intensity of *P. falciparum* transmission yet the Fulani are less suspectable to *P. falciparum* infection (154). DCs from malaria infected children of the Dogon community expressed lower levels of HLA-DR and CD86 on their DCs, while the frequency of BDCA-2+ pDCs and BDCA-3+ cDC1 increased compared to uninfected counterparts. Infected children from the Fulani community exhibited higher levels of HLA-DR and CD86 on their DCs but had a lower number of circulating BDCA-2+ pDCs and BDCA-3+ cDC1 compared to their uninfected counterparts (150). The study also showed that infected children from the Fulani community retained their ability to produce IFN-γ after their PBMC were stimulated with specific TLR ligands at levels that were similar to those of uninfected children. The Dogon children, on the other hand, had low levels of cytokine produced due to TLR impairment which increased parasite burden and development of malaria symptoms (150). This showed that *P. falciparum* infection resulted in altered DC activation with reduced response to TLR agonists in Dogon children, while in the Fulani children, DC activation and TLR responses were unaffected.

The increase in the number of circulating BDCA-2+ pDCs and BDCA-3+ cDC1 during malaria infection has been attributed to an increase in the amounts of FMS-like tyrosine kinase 3 (Flt3) ligand (Flt3-L) (155). Flt3 is highly expressed on hematopoietic progenitor cells, but the expression is lost as cells commit to lymphoid and myeloid progenitor cells, which gives rise to the various cell lineages but its expression on DCs remains. Flt3 receptor tyrosine kinase and its ligand Flt3-L are known to be key in the development of dendritic cells and maintenance of their numbers (156, 157). Flt3-L production increases during a malaria episode as mast cells become activated and release membrane bound Flt3-L into circulation resulting in an increase in the number of pDCs and CD1c (155).

A few studies have looked at the function of DCs in adults during a malaria episode. A study in Thailand found that adults with both severe and mild malaria had a decreased number of TLR2 expressing cDCs circulating in the periphery and a lower surface expression of TLR9 on pDCs but an increase in the surface expression of TLR2 on cDCs compared with healthy controls (158). There was also a marked reduction in the number of circulating pDCs, this could be attributed to their migration to the secondary lymph nodes, and an increase in serum levels of IFN-α (159). A study conducted in Papua found that adults with acute *P. falciparum* malaria had a reduced number of circulating pDCs and cDCs, but higher numbers of immature DCs that were HLA-DR+CD11c–CD123– (5). Interestingly both pDCs and cDCs from infected participants were apoptotic as seen by Annexin-V binding. The DCs also expressed low levels of HLA-DR and costimulatory molecules and were unable to adequately capture antigen, resulting in reduced ability to prime naive CD4 T cells (5). These studies are therefore consistent with a role for malaria infection in reducing the number of circulating DCs and their function in antigen presentation and T cell activation.

Controlled human infection model (CHMI) have also been used to assess the function of BDCA-1+ cDC2 and pDCs at varying doses of *P. falciparum* (160). Healthy volunteers were enrolled into two cohorts; one cohort was inoculated with 150 iRBCs and the other 1,800 iRBCs, participants were treated once parasitaemia reached ≥ 1,000 parasites/ml (160, 161). The expression levels of HLA-DR on BDCA-1+ cDC2 and pDCs in both cohorts were significantly reduced at peak parasitaemia and this effect was still evident on BDCA-1+ cDC2 24 h after anti-malarial treatment. The cohort inoculated with a higher dose of iRBC had a reduced number of circulating BDCA-1+ cDC2 which was attributed to apoptosis of the DCs during the course of the infection, this was evident by the upregulation of caspase-3 (160). The BDCA-1+ cDC2 from this cohort had a defective phagocytic capacity and there was a positive association between HLA-DR expression and phagocytic capacity (160). pDCS on the other hand expressed low levels of CD123 at peak parasitaemia in both cohorts which persisted 24 h after anti-malarial treatment. The number of pDCs in circulation significantly reduced in the s iRBC cohort, this was due to apoptosis of pDCs during the course of infection (161). At peak parasitaemia DCs from the 1,800 iRBC cohort were restimulated *ex vivo* with TLR ligands and their response measures. On re-stimulation with TLR1/2, TLR4, and TLR7, BDCA-1+ cDC2 failed to upregulate HLA-DR and CD86 but increased TNF secretion (160). While re-stimulation of pDCS with TLR7 and TLR9 resulted in upregulation of HLA-DR, CD123, CD86 on their surface and an increased secretion of IFN-α (161). This shows that malaria infection in naive individuals results in impairment of cDC function but not pDCs function. Indicating that pDCs may play a role during malaria infection and further studies are needed to deduce its role. The altered BDCA-1+ cDC2 also contributed to hampering effector T cells functions, allowing an increase of parasite burden (160).

The various studies above have shown that DC phenotype is altered during a malaria episode resulting in impaired ability to upregulate HLA-DR and the costimulatory molecules CD86 (150, 153, 160). This altered DC phenotype has a reduced phagocytic capacity which impairs its ability to process antigens (160) and adequately stimulate allogeneic T cells (5, 153). The parasite also modulates TLR signaling thereby affecting cytokine secretion (150, 160) resulting in severe pathology. In children, there seems to be a notable increase in the number of circulating BDCA-3+ cDC1s during a malaria episode (150, 153, 155), which was attributed to increases in serum levels of Flt3-L (155), but this effect was not observed in children from Papua (162). In both children and adults, there was a decrease in the number of circulating DCs which was attributed to increased DC apoptosis (5, 159, 161) but also increased DC migration to secondary lymphoid organs may also play a role in reduction on peripheral blood DC numbers. The decrease in peripheral numbers of DCs also corresponded with an increase in IL10 and TNF-α (5, 149, 153), which may play a role in DC loss of function and suppression of T cell function. In these studies, DC function was altered regardless of the severity of malaria infection. The DC phenotype seen in the acute infection in the CHMI study (160), was similar to those seen in naturally exposed individuals, and repeated infection, in naturally exposed individuals, could lead to sustained downregulation of DC function that may impact negatively on the immunity of an individual.

IN VIVO MOUSE MODELS OF MALARIA

Mouse models have been extensively used to study DC-*Plasmodium* interaction. *In vitro* interaction of *P. chabaudi* schizonts with mouse bone marrow derived DCs resulted in an increase in the secretion of tumor necrosis factor-α(TNF-α), IL-6, and IL-12p40 and IL-12p70 (152). In mice injected with *P. chabaudi*, DCs had fully functional cytokine production 6 days after challenge with *Plasmodium* parasite (163). Further studies demonstrated DCs were able to upregulate co-stimulatory molecules CD40, CD54, CD86 (164) during acute infection, and were able to migrate into T cell areas in the spleen (165). Other studies with *P. chabaudi* show that during initial stages of murine erythrocyte infection, CD8+ DCs are activated by infected erythrocytes as they expressed high levels of MHC II and costimulatory molecules and initiated a Th1 type of response. This response is short lived as the CD8+ DCs undergo apoptosis and are soon replaced by CD8- DCs with lower expression levels of costimulatory molecules and MHC II (166).

Consistent with the studies above, Millington et al. (8) showed that DCs isolated from the spleen of mice 4 days after *P. chabaudi* infection were moderately activated as they upregulated surface expression of CD40, CD80, and CD86. However, during convalescence (days 12 and 21 post-infection), DC did not upregulate costimulatory molecules and were refractory to stimulation with LPS or CD40L. When mice infected with *P. chabaudi* were immunized with ovalbumin (OVA) antigen and LPS, they produced significantly lower levels of OVA-specific IgG compared with uninfected immunized mice, however, this effect was only seen when immunized at days 12 and 21 post infection (not day 4). Thus, initial malaria infection in mice does seem to cause DC activation; however DCs enter a refractory state in following the initial peak of parasitaemia. Similar to convalescent DCs, *in vitro* bone marrow derived DCs pre-exposed to *P. chabaudi* were unable to increase expression levels of MHC II and co-stimulatory molecules CD40, CD80, and CD86, and LPS stimulation of these DCs was unable to increase their expression (8).

Further work suggested that hemozoin could also modulate DC function which resulted in impairment of T cell and B cell function. Hemozoin treated DCs retained their capacity to process antigen and present them on MHC class II to naive CD4 T cells. Thus providing the essential signal 1 (peptide-MHC complex) via the T cell receptor (TCR) but these DCs were unable to form stable long lasting clusters with naive T cells, resulting in the generation of dysfunctional T cells (8, 104). These dysfunctional T cells failed to proliferate and produce adequate amount of effector cytokines (IL-2, IL-5, IL-10, IFNγ) (8), and were unable to migrate to B cell areas in the lymph nodes to aid in B cell proliferation and antibody production (8, 104). The short interactions and lack of large clustering observed are known to interfere with the generation of Tfh cells as long sustained DC-T cells interaction is required for commitment of naive CD4 T cells to Tfh cells (167). It is possible the dysfunctional DCs can lead to the generation of exhausted T cells, as a result of the short time of antigen presentation to the T cells in the absence of adequate co-stimulation. The dysfunctional T cells could also lead to the generation of atypical memory B cells which are normally associated with malaria episodes.

Dendritic cells have been shown to play a vital role in the survival of mice during a lethal infection with *P. yoelii*. Wykes et al. (168) showed that DCs from mice infected with non-lethal *P. yoelii* infection were fully functional APC and maintained their ability to stimulate T cells, unlike DCs from lethal *P. yoelii* infection which were not functional. DCs from mice infected with the non-lethal parasite were adoptively transferred into naive mice, which were then infected with lethal infection *P. yoelii*. These DCs were able to control parasitaemia and aid in survival of the mice by secreting IL-12 (168). This could in part explain the difference in malaria outcomes observed in natural infections.

DOWNSTREAM EFFECT OF DC DYSREGULATION

Collectively, the studies above support the hypothesis that during the blood stage of *Plasmodium* infection, DC function is dysregulated resulting in phenotypically altered DCs that are unable to appropriately activate naive CD4 cells. Furthermore, the failure by naive CD4 T cells to differentiate into CD4 follicular helper T cells results in a failure of B cell help and reduced humoral immunity. The phenotype of the resulting T cell population is unclear; however, *P. falciparum* infection has

been associated with increased expression of the T cell inhibitory receptor programmed cell death-1 (PD-1) on CD4 T cells. This was observed in a cohort of children in Mali (169) and Kenya (7), during an ongoing *P. falciparum* infection. Apart from an increase in the expression of PD-1 on CD4 T cells, *P. falciparum* infection was seen to drive an increase in the frequency of atypical memory B cells which was as a result of the exhausted T cell phenotype (7).

Butler et al. (169) using non-lethal *P. yoelii* infections, also showed that prolonged infection resulted in dysfunctional parasite specific CD4 T cells that expressed exhaustion markers PD-1 and lymphocyte-activation gene-3 (LAG-3) (169). These inhibitory ligands worked in synergy to inhibit T cell function during the *Plasmodium* infection. The ability of CD4 T cells to produce cytokines deteriorated with prolonged infection, while dual blockade of PD-1 and LAG-3 with monoclonal antibodies restored the number of parasite specific CD4 T cells and their ability to secrete cytokines. It also resulted in an increase in the number of CD4 Tfh cells and plasmablasts, thus improving the anti-parasite humoral response in *P. yoelii* infected mice (169). These studies show that that malaria induces T cell exhaustion and that PD-1 plays a role in the pathogenesis of malaria.

Apart from the upregulation of the T cell inhibitory receptors programmed cell death-1 (PD-1) and Lymphocyte Activation Gene 3 (LAG3), malaria infection upregulates the production of IFN-γ and IL-10 on CD4 T cells (170, 171). This creates a suppressive environment that polarizes CD4 T cells toward IFN-γ producing Th1 like lineage, suppressing induction of Th2 and Tfh, which are vital in B cell response. This polarization occurs after a single malaria episode and may affect subsequent parasite exposures

T cell exhaustion can be due to persistent antigen exposure, resulting in sustained TCR stimulation by dysfunction DCs, leading to sustained upregulation of PD-1 (172, 173). The inhibitory PD1 signal on T cells works by inhibiting downward signals from TCR and costimulatory molecules and initiates transcription of inhibitory genes (**Figure 2B**) (174–176). Cytokine signaling also plays a role in T cell exhaustion. Malaria induces secretion of IL-10 from DCs, providing an immunosuppressive environment that skews the development of CD4 T cells and dual blocking IL-10 and PD-1 signaling in mice restores T cell function (177). Transcriptional profile of exhausted T cells greatly varies from effector and memory T cells, indicating that exhaustion is a unique state of T cell differentiation (178–180), that is regulated by the master transcription factor TOX (181). The signaling pathways that lead to the differentiation of exhausted T cells and expression of TOX are yet to be known.

There are still gaps in our current understanding of the intracellular mechanism of PD1 signaling and what its target genes are. The molecular events initiated by downstream IL-10 signaling that shape T cell exhaustion are yet to be known.

CONCLUSION

The population in malaria endemic areas are known to have a reduced immune response against vaccines (2, 3), and ongoing malaria infections in these individuals reduce pre-existing adaptive immune responses (101). The evidence presented above strongly indicates this is due to dysfunctional DCs that fail to prime effective T cell responses, thus affecting immune responses. There is a potential gap is in our understanding of the effect of antimalarial drugs on the phenotype, function and numbers of DCs and T cells. Whether antimalarial treatment restores DC-T interaction is an area of research is yet to be explored.

This information could address a significant public health challenge in administering malaria vaccines and other vaccines in malaria endemic areas. Malaria infection also induces CD4 T cell exhaustion, through upregulation of negative regulatory molecules such as PD-1 and LAG3 (7, 169), which dampen immune responses. How T cell exhaustion is induced in malaria is still unknown as there is no evidence in literature explaining how and where these cells arise, and if dysfunctional DCs play a role in this, and how the cytokine environment during a malaria episode influence T cell exhaustion. There is a need to better understand the interaction between DC-T cell, the cellular and molecular signals that are involved in the formation of this immune synapse and how malaria affects this interaction. This will aid in developing novel methods that will target the affected molecular pathways and restore DC-T cell interaction and function.

AUTHOR CONTRIBUTIONS

RO wrote the first draft of the manuscript, which was reviewed and edited by TO, FN, PG, and JB. All authors contributed to the article and approved the submitted version.

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Household Contacts of Leprosy Patients in Endemic Areas Display a Specific Innate Immunity Profile

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Leprosy is a chronic infectious disease, caused by *Mycobacterium leprae*, that can lead to severe life-long disabilities. The transmission of *M. leprae* is continuously ongoing as witnessed by the stable new case detection rate. The majority of exposed individuals does, however, not develop leprosy and is protected from infection by innate immune mechanisms. In this study the relation between innate immune markers and *M. leprae* infection as well as the occurrence of leprosy was studied in household contacts (HCs) of leprosy patients with high bacillary loads. Serum proteins associated with innate immunity (ApoA1, CCL4, CRP, IL-1Ra, IL-6, IP-10, and S100A12) were determined by lateral flow assays (LFAs) in conjunction with the presence of *M. leprae* DNA in nasal swabs (NS) and/or slit-skin smears (SSS). The HCs displayed ApoA1 and S100A12 levels similar to paucibacillary patients and could be differentiated from endemic controls based on the levels of these markers. In the 31 households included the number (percentage) of HCs that were concomitantly diagnosed with leprosy, or tested positive for *M. leprae* DNA in NS and SSS, was not equally divided. Specifically, households where *M. leprae* infection and leprosy disease was not observed amongst members of the household were characterized by higher S100A12 and lower CCL4 levels in whole blood assays of HCs in response to *M. leprae*. Lateral flow assays provide a convenient diagnostic tool to quantitatively measure markers of the innate immune response and thereby detect individuals which are likely infected with *M. leprae* and at risk of developing disease or transmitting bacteria. Low complexity diagnostic tests measuring innate immunity markers can therefore be applied to help identify who should be targeted for prophylactic treatment.

Keywords: innate immunity, lateral flow test, diagnostics, *M. leprae*, UCP-LFA, leprosy

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INTRODUCTION

Leprosy is a debilitating disease that is one of the leading causes of long-term nerve damage worldwide (1). Multidrug therapy (MDT) effectively kills *Mycobacterium leprae,* the causative agent of leprosy, providing an effective cure when treatment is initiated timely (2, 3). To achieve elimination of leprosy, however, it is vital to not only treat adequately and timely but also to prevent

transmission (4). The stable new case detection rates in many leprosy endemic countries (5) indicate that MDT insufficiently reduces transmission of *M. leprae*. Recognition of the often subtle cardinal clinical signs is of major importance for leprosy diagnosis (6). The declaration of the WHO in 2000 that leprosy had been eliminated as a public health problem (7), however, caused a reduction of leprosy control activities. The reduced intensity in case detection activities and training in the diagnosis and treatment of leprosy results in many cases that remain undetected for several years (8), allowing the transmission of *M. leprae* to continue.

Contacts close to leprosy patients have a higher risk of acquiring the infection, especially when the patients carry high bacillary loads (9–11). Fortunately, the majority of exposed individuals is naturally immune to *M. leprae* infection (12). Host immunity also determines the clinical phenotype of leprosy, ranging from paucibacillary (PB) patients with a strong proinflammatory response (Th1/Th17) leading to bacterial control to multibacillary (MB) patients with an anti-inflammatory immune response (Th2) producing large quantities of antibodies but unable to control the bacteria (13, 14). In the innate immune response macrophages are critical mediators that define the course of *M. leprae* infection and clinical outcome. In PB patients IL-15 induces antimicrobial activity and the vitamin D-dependent antimicrobial program in macrophages restricting bacterial dissemination (proinflammatory M1 macrophages) (15). In contrast, in MB patients a scavenger receptor program is induced by IL-10, leading to foam cell formation by increased phagocytosis of mycobacteria and oxidized lipids, and persistence of *M. leprae* (anti-inflammatory M2 macrophages) (16, 17).

Markers of the innate immune response can thus be helpful to identify *M. leprae* infected individuals who are prone to develop leprosy disease and thereby, since they are unable to kill and remove *M. leprae*, contribute to the ongoing transmission. No practical tools are yet available to identify individuals that should be prioritized for prophylactic treatment. Recently, biomarkers for leprosy and *M. leprae* infection were identified (18, 19), including serum proteins that play a role in innate immunity. For example, S100A12 is required to decrease *M. leprae* viability in infected macrophages (20). CCL4 and IP-10 attract innate immune cells such as natural killer (NK) cells and monocytes, whereas IL-1Ra-stimulated monocytes turn into M2 macrophages that produce high levels of the anti-inflammatory cytokine IL-10 (21).

Two other identified biomarkers (19) that play a role in the innate immune system were contrasting acute phase proteins: anti-inflammatory ApoA1 and pro-inflammatory CRP. ApoA1 inhibits the recruitment of monocytes and macrophage chemotaxis (22), whereas CRP can recognize pathogens and activate the classical complement pathway (23). Together with αPGL-I IgM, the well-established biomarker for MB leprosy (24), the identified biomarkers were implemented in quantitative up-converting phosphor lateral flow assays (UCP-LFAs) (19). These user-friendly tests are applicable in resource-limited settings, essential for diagnostic tools in large-scale contact screening of leprosy contacts, and provide quantitative results. The latter allows monitoring of drug treatment as well as discriminating high from low responders.

Previously, we analyzed nasal swabs (NS) and slit-skin smears (SSS) of household contacts (HCs) of MB leprosy patients with high bacillary loads for the presence of *M. leprae* DNA (25). Here we analyzed the same individuals to examine the correlation of the presence of *M. leprae* DNA with the levels of innate immune markers. *M. leprae* DNA in NS indicates colonization of the HC with the bacterium, but not invasion of the tissue. Detection of *M. leprae* DNA in SSS does indicate that a HC is infected. In this study, levels of ApoA1, CCL4, CRP, IL-1Ra, IL-6, IP-10, αPGL-I IgM, and S100A12 were determined by UCP-LFAs in supernatants of 24 h *M. leprae* antigen-stimulated whole blood assays (WBA) addressing newly diagnosed MB patients with a high bacteriological index (BI) and their HCs in Bangladesh.

MATERIALS AND METHODS

Study Participants

The cohort used in this study originates from four districts in Bangladesh (Nilphamari, Rangpur, Panchagar, and Thakurgaon) and has been extensively described previously (25). The prevalence of leprosy in these districts was 0.9 per 10,000 and the new case detection rate 1.18 per 10,000 (Rural health program, the leprosy mission Bangladesh, yearly district activity report 2018).

Between July 2017 and May 2018, newly diagnosed leprosy patients (index case; $n = 31$) with BI $\geq$2 and between 3 and 15 HCs per index case ($n = 279$) were recruited (25). Leprosy was diagnosed based on clinical and bacteriological observations and classified as MB or PB as described by the WHO (5) and the BI was determined. HCs were examined as well for signs and symptoms of leprosy upon recruitment and followed up yearly for surveillance of new case occurrence for $\geq$24 months after sample collection.

Control individuals without known contact to leprosy or TB patients and without clinical disease symptoms from the same leprosy endemic area (EC) were included and assessed for the absence of clinical signs and symptoms of leprosy and TB. Staff of leprosy or TB clinics were excluded as EC.

Household Contacts

The coding system used to describe physical and genetic distance of contacts from the patient has been extensively described previously (26). In short, four categories of physical distance are relevant for this study:

- KR: contacts living under the same roof and the same kitchen
- K: contacts living under a separate roof but using the same kitchen
- R: contacts living under the same roof, not using the same kitchen
- N1: next-door neighbors

In this study the KR and R group were considered as one group.

For genetic distance seven categories were defined: spouse (M), child (C), parent (P), sibling (B), other relative (O), relative

in-law (CL, PL, BL, or OL), and not family related (N). CL, PL, and OL were considered as one group in this study, referred to by OL.

Ethics

This study was performed according to the Helsinki Declaration (version Fortaleza, Brazil, October 2013). The studies involving human participants were reviewed and approved by the Bangladesh Medical Research Council/National Research Ethics Committee (BMRC/NREC/2010-2013/1534). Participants were informed about the study-objectives, the samples and their right to refuse to take part or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. All patients received treatment according to national guidelines.

Sample Collection

SSS from the earlobe and NS were collected for detection of *M. leprae* DNA as described previously (25). For the WBA, 4 ml venous blood was drawn and 1 ml was applied directly to a microtube precoated with 10 μg *M. leprae* whole cell sonicate (WCS) or without stimulus (Med). After 24 h incubation at 37°C the microtube was frozen at −20°C, shipped to the LUMC and stored at −80°C until further analysis.

DNA Isolation and RLEP PCR/qPCR

DNA isolated from the NS and SSS was used to perform RLEP PCR and qPCR as described previously (25). Presence of *M. leprae* DNA was considered if a sample was positive for RLEP qPCR with a Ct lower than 37.5 or was positive for RLEP PCR at least in two out of three independently performed PCRs to avoid false positives.

UCP-LFAs

Levels of αPGL-I IgM, CRP, IP-10, S100A12, ApoA1, IL-6, IL-1Ra, and CCL4 in WBA supernatant were analyzed using UCP-LFAs. αPGL-I IgM, CRP, IP-10, S100A12, and ApoA1 UCP-LFAs have been described previously (18, 19). IL-6, IL-1Ra, and CCL4 UCP-LFAs were produced similarly, with a Test line of 200 ng MQ2-39C3 (IL-6; BioLegend, San Diego, USA), AF280 (IL-1Ra), and clone 24006 (CCL4) (R&D systems, Minneapolis, USA) and a Flow Control line with 100 ng Goat-anti-Rat (IL-6; R5130, Sigma-Aldrich), Goat-anti-Mouse (IL-1Ra; M8642; Sigma-Aldrich), and Rabbit-anti-Goat (CCL4; G4018, Sigma-Aldrich). Complementary antibodies were conjugated to the UCP particles, MQ2-13A5 (BioLegend, San Diego, USA), clone 10309 (IL-1Ra), and AF-271-NA (CCL4) (R&D systems, Minneapolis, USA). Yttrium fluoride upconverting nano materials (200 nm, NaYF4:Yb3+,Er 3+) functionalized with polyacrylic acid were obtained from Intelligent Material Solutions Inc. (Princeton, New Jersey, USA).

To perform the UCP-LFAs WBA supernatant was diluted 5-fold (IP-10, IL-1Ra and CCL4), 50-fold (IL-6, αPGL-I IgM and S100A12), 500-fold (CRP) and 5,000-fold (ApoA1) in high salt buffer (100 mM Tris pH 8, 270 mM NaCl, 1% (w/v) BSA, 1% (v/v) Triton X-100). As WCS stimulation does not affect the levels of ApoA1, CRP, and αPGL-I IgM these three markers were only determined in medium. Strips were analyzed using a UCP dedicated benchtop reader (UPCON; Labrox, Finland). Results are displayed as the ratio value between Test and Flow-Control signal based on relative fluorescence units (RFUs; excitation at 980 nm and emission at 550 nm) measured at the respective lines.

Statistical Analysis

GraphPad Prism version 8.1.1 for Windows (GraphPad Software, San Diego CA, USA) was used to perform Mann-Whitney *U*-tests, Kruskal-Wallis with Dunn's correction for multiple testing, Wilcoxon matched-pairs signed rank test, plot receiver operating characteristic (ROC) curves, and calculate the area under curve (AUC). The Pearson correlation coefficient and the corresponding *p*-values and heatmap were also determined using GraphPad Prism.

RESULTS

M. leprae DNA in Nasal Swabs/Slit-Skin Smears and the Occurrence of Leprosy in HCs

The presence of *M. leprae* DNA in NS and SSS of HCs was assessed in 31 households of MB index cases with BI ≥2 (25) (**Figure 1**). Out of 279 HCs, 29 were diagnosed with leprosy upon first physical investigation at intake, and four were diagnosed with PB leprosy during follow-up. Of the patients diagnosed at intake the majority (93%) had a low bacillary load: 22 were PB and seven were MB, of whom five with BI 0 (MB/BT) and two with BI ≥4 (**Supplementary Figure 1**). The HCs diagnosed with leprosy at intake (DevLep) were not evenly distributed over the different households: in 14 households none of the HCs had developed leprosy, whereas in the other 17 households, 9–42% suffered from leprosy (**Figure 1**). Applying previous results on the presence of *M. leprae* DNA (25), indicated that in 10 households *M. leprae* DNA was not detected in any of the HCs in NS and in 13 households all HCs were negative in the SSS. Of the households where *M. leprae* DNA was detected, percentages of colonization varied from 7 to 100% (NS) and for infection from 10 to 66% (SSS; **Figure 1**). The proportion of *M. leprae* DNA presence in NS or SSS and identified leprosy in HCs upon first physical screening thus varies between households even if the index cases have similarly high bacillary loads.

ApoA1 and S100A12 Levels Differentiate HCs From EC

Levels of αPGL-I IgM, CRP, IP-10, S100A12, ApoA1, IL-6, IL-1Ra, and CCL4 were determined by UCP-LFA in WBA supernatant. Levels of these eight markers in patients ($n = 62$; 38 MB and 24 PB), HCs ($n = 244$) and EC ($n = 20$) without known contact to leprosy patients were compared. Stimulation with *M. leprae* WCS had a significant impact on the CCL4, IL-1Ra, and IL-6 levels (**Supplementary Figure 2**). Significant differences between the groups were observed for αPGL-I IgM, $S100A12_{Med}$, $S100A12_{WCS}$, ApoA1, and CRP (**Figure 2A**). Compared to EC, the AUC values for αPGL-I IgM and CRP were significant only for MB patients, whereas ApoA1 and S100A12

A

Household					Index case					
HH ID	Contacts (n)	% DevLep	%NS+	%SSS+	Gender	Age	BI	RLEP+	SSS (Ct)	NS (Ct)
HH1	5	20	20	0	Male	19	6	SSS+NS	16,25	20,06
HH2	11	0	55	36	Female	46	6	SSS+NS	16,81	21,24
HH3	8	13	25	63	Male	26	6	SSS+NS	17,98	21,21
HH4	10	0	10	10	Male	45	6	SSS+NS	18,40	22,73
HH5	0	NA	NA	NA	Male	48	6	SSS+NS	18,94	24,20
HH6	9	11	0	22	Male	45	6	SSS+NS	19,04	24,95
HH7	8	0	0	0	Male	30	6	SSS+NS	19,17	24,65
HH8	10	10	40	50	Male	33	5	SSS+NS	20,73	23,86
HH9	8	0	50	13	Male	25	6	SSS+NS	21,21	23,31
HH10	12	25	25	0	Male	34	6	SSS+NS	21,65	30,79
HH11	7	0	0	14	Male	31	5	SSS+NS	21,76	33,18
HH12	10	20	20	20	Male	38	6	SSS+NS	22,40	28,58
HH13	13	0	0	0	Male	48	5	SSS+NS	22,55	29,89
HH14	4	0	25	25	Male	36	5	SSS+NS	22,67	31,57
HH15	12	0	33	0	Male	67	5	SSS+NS	22,96	27,78
HH16	3	33	33	66	Female	12	6	SSS+NS	23,05	35,83
HH17	6	0	0	33	Male	27	6	SSS+NS	23,13	28,19
HH18	15	0	7	0	Male	35	6	SSS+NS	23,19	24,82
HH19	5	40	100	0	Male	45	6	SSS+NS	23,25	21,15
HH20	12	42	42	33	Female	17	6	SSS+NS	23,71	26,27
HH21	10	10	30	0	Female	33	5	SSS+NS	24,33	23,27
HH22	11	9	0	0	Male	32	6	SSS+NS	25,35	29,21
HH23	7	14	57	14	Male	36	5	SSS+NS	25,59	26,70
HH24	9	0	0	0	Male	50	6	SSS+NS	25,78	31,75
HH25	14	21	0	0	Male	50	5	SSS+NS	26,21	30,46
HH26	10	0	10	0	Male	30	6	SSS+NS	26,27	20,70
HH27	9	0	22	11	Female	36	4	SSS+NS	27,84	32,31
HH28	11	18	0	18	Male	48	2	SSS	32,58	Undetermined
HH29	12	0	17	25	Male	26	6	NS	NA	33,66
HH30	10	40	30	10	Male	56	4	None	NA	Undetermined
HH31	6	17	0	0	Female	30	4	NS	NA	29,25

B

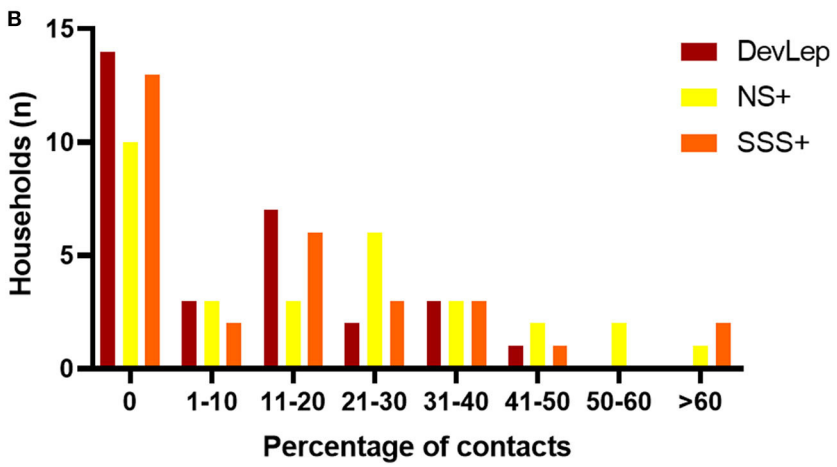

FIGURE 1 | Percentage of *M. leprae* DNA positive nasal swabs/slit-skin smears and occurrence of leprosy in contacts per household. **(A)** Table indicates the number of household contacts per index case, the percentage of contacts that were diagnosed with leprosy during contact screening (%DevLep) and the percentage of contacts with *M. leprae* DNA detected in nasal swabs (%NS+) and slit-skin smears (%SSS+). The characteristics of the index case of each household (HH) are also indicated in this table. RLEP+ indicates whether *M. leprae* DNA was detected in the NS or SSS of the index case, the corresponding Ct values are indicative of the amount of *M. leprae* bacilli in NS and SSS. A low Ct value corresponds to high amounts of bacteria. BI, bacteriological index; NA, Not applicable. **(B)** On the *x*-axis the

(Continued)

FIGURE 1 | percentage range of household contacts (HCs) diagnosed with leprosy during contact screening (DevLep; dark red bars), that were *M. leprae* DNA positive in nasal swabs (NS+; yellow bars) or slit-skin smears (SSS+; orange bars) is indicated. The *y*-axis depicts the number of households for the percentage range indicated on the *x*-axis. The number of households within each percentage range was determined using the data table from **(A)**.

levels significantly differed in both MB and PB patients. In HCs, however, the levels of S100A12 were comparable to those in (MB and PB) patients with similar AUCs (ranging from 0.85 to 0.91; **Figure 2B**). Interestingly, the difference in ApoA1 levels between EC was more profound for HC (AUC:0.81; $p < 0.0001$) than for PB (AUC:0.76; $p = 0.0039$) or MB patients (AUC: 0.7; $p = 0.0126$). As described for other cohorts previously (18), MB patients can be discriminated from HCs based on αPGL-I IgM ($p < 0.0001$) and CRP ($p = 0.0024$), but these markers cannot differentiate PB patients from HCs with similar rates of *M. leprae* DNA presence in NS and SSS (25). These data thus indicate that PB patients and HCs respond similarly to *M. leprae*.

S100A12 and CCL4 Response Is Associated With the Occurrence of Leprosy in Households

The relationship between disease and infection/colonization status in households was examined into more detail by determining the correlation between the immune markers and the percentage of HCs with detectable *M. leprae* DNA in NS (%NS) and SSS (%SSS) or diagnosed with leprosy (%DevLep) (**Figure 3A**). A highly significant ($p < 0.0001$) positive correlation was identified for the %DevLep with $CCL4_{WCS}$ and a negative correlation for %SSS with $S100A12_{Med}$ and $S100A12_{WCS}$ (**Supplementary Table 1**). For a subset of individuals qPCR Ct values were available indicative of the quantity of *M. leprae* DNA in NS ($n = 105$) or SSS ($n = 71$). These Ct values showed an inverse correlation with αPGL-I IgM antibodies in this cohort, indicating a strong positive correlation between the amount of *M. leprae* and the PGL-I antibody titer (25). For $IL\text{-}1Ra_{Med}$/$IL\text{-}1Ra_{WCS}$ and inversely for CRP, a significant correlation was observed with the Ct values for both NS and SSS as well (**Supplementary Table 1**).

A cross-sectional analysis was performed to compare households in which HCs developed leprosy to households where this was not observed. The same analysis was performed for households where *M. leprae* DNA was present in NS or SSS of HCs. In households where *M. leprae* DNA was detected in NS significantly lower levels of $S100A12_{Med}$ ($p < 0.0001$) and $S100A12_{WCS}$ ($p = 0.0005$) and higher levels of $IL\text{-}1Ra_{WCS}$ were observed (**Figure 3B**). S100A12 levels were also significantly lower in households where *M. leprae* DNA was detected in SSS (**Figure 3C**; $p < 0.0001$). CCL4 levels were higher in these households, especially in response to *M. leprae* WCS ($p < 0.0001$). Higher levels of $CCL4_{WCS}$ were also observed in the households where HCs of the primary index case were diagnosed with leprosy upon first physical investigation at intake ($p = 0.0002$) as well as increased levels of CRP ($p = 0.025$; **Figure 3D**).

The levels of CCL4 and S100A12 showed a significant result in both the correlation and cross-sectional analysis, indicating an association of these markers with leprosy and/or *M. leprae* infection among HCs.

M. leprae Colonization in HCs Correlates With Physical Distance to the Index Case

To examine the influence of the characteristics of the index case (all MB patients with high bacillary loads) on the development of leprosy and *M. leprae* colonization (NS) or infection (SSS) in HCs, a correlation and cross-sectional analysis was performed (**Supplementary Figure 3**). Cross-sectionally, higher $S100A12_{Med}$ levels were observed in index cases without detectable *M. leprae* DNA in NS of their HCs ($p = 0.035$). No other significant differences were observed in index cases for the other markers nor in the amount of bacteria in SSS or NS. Thus, characteristics of the index case in this cohort have little influence on the observed differences between the households (**Figure 1**).

The influence of genetic relationship and physical distance of HCs to the index case was also examined. HCs were stratified by genetic distance against the percentage of leprosy and *M. leprae* DNA presence in NS and SSS in these groups (**Figure 4**). Development of leprosy was most frequently observed in spouses (37%), followed by siblings (23%) and siblings in law (17%) (**Figure 4A**). Spouses also showed the highest frequency of *M. leprae* presence in NS and/or SSS (58%), followed by children (42%), and parents (41%) (**Figure 4B**). Spouses, children, and parents live in the closest proximity of patients (**Figure 4C**; living under the same roof or sharing a kitchen) and thus have the highest level of exposure. Physical distance indeed correlated significantly ($p = 0.003$; $R^2 = 0.8$) with the $\%NS_{Pos}$ (colonization), though this was not observed for the development of leprosy in HCs ($p = 0.07$; $R^2 = 0.44$).

The levels of the innate immune markers were also stratified by genetic distance. Based on the median levels of the assessed markers, the HC groups that were diagnosed with leprosy clustered apart from the HC groups that did not show symptoms of disease (**Figure 5**). Across the groups with different genetic distance to the index case, similar innate immune mechanisms seem to play a role in the development of leprosy in HCs. Additionally, the index case group clustered apart from all HC groups rendering the assessed markers useful for leprosy diagnostics.

DISCUSSION

To examine the link between innate immunity and *M. leprae* colonization/infection in HCs, immune markers were assessed in 24 h *M. leprae* antigen-stimulated WBAs by UCP-LFAs. Even though all HCs were exposed to comparable levels of *M. leprae*, as all 31 index cases were MB patients with BI ≥2, there was a difference in the percentage of *M. leprae* DNA presence in NS/SSS and the occurrence of leprosy cases between households.

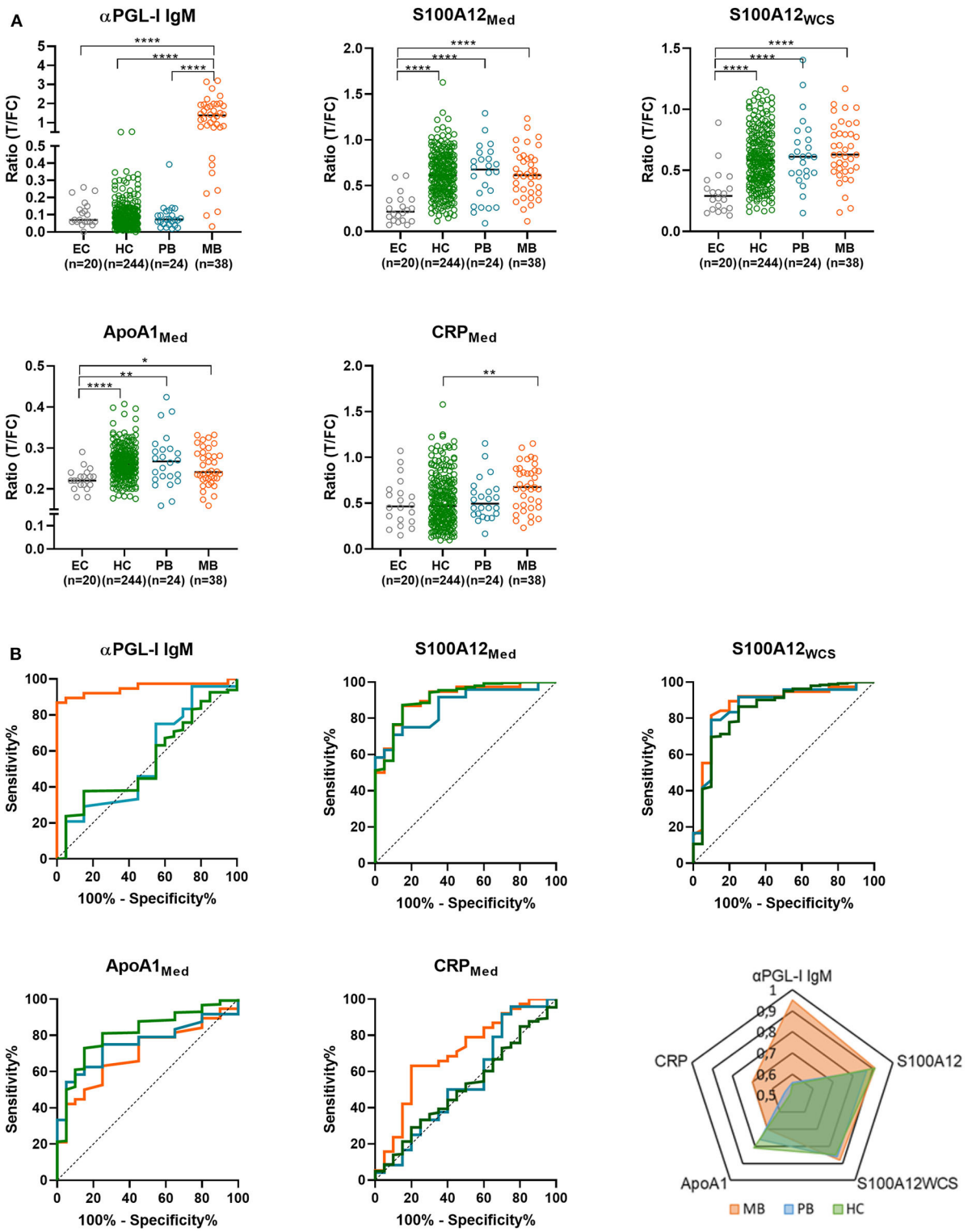

FIGURE 2 | Differentiation of leprosy patients and household contacts (HC) from endemic controls (EC) by immune markers. Whole blood without stimulus (Med) or stimulated with *M. leprae* whole cell sonicate (WCS) was frozen after 24 h. Levels of 8 proteins (αPGL-I IgM, S100A12, ApoA1, CCL4, IP-10, IL-6, IL-1Ra, and CRP) were assessed by up-converting phosphor lateral flow assays (UCP-LFAs) in these whole blood assay supernatants for 31 households of index cases with multibacillary (MB) leprosy (bacteriological index ≥2). **(A)** UCP-LFA ratio values were calculated by dividing the peak area of the test line (T) by the peak area of the flow control line (FC; *y*-axis). As ratio values are marker dependent the *y*-axis scale differs per marker. The levels of MB (orange circles) and paucibacillary (PB; blue circles) patients, household contacts (HC; green circles) and endemic controls (EC; gray circles) were compared using the Kruskal-Wallis test with Dunn's correction for

(Continued)

FIGURE 2 | multiple testing. The data of CCL4, IP-10, IL-6, and IL-1Ra were not shown as no significant differences were observed in the levels of these proteins between groups. *P*-values: $*p \leq 0.05$, $**p \leq 0.01$, $****p \leq 0.0001$. **(B)** Receiver operating characteristic (ROC) curves were computed comparing the levels of αPGL-I IgM, CRP, S100A12, ApoA1 in multibacillary (MB) /paucibacillary (PB) patients and HC to EC. These levels were determined by up-converting phosphor lateral flow assays in supernatant of 24 h *M. leprae* antigen-stimulated whole blood assays (WBA; medium = Med, *M. leprae* whole cell sonicate = WCS). A summary of the areas under the curve (AUC) for MB (orange), PB (blue) and HC (green) is depicted in the spider plot showing the markers in which significant differences were observed (lower right panel).

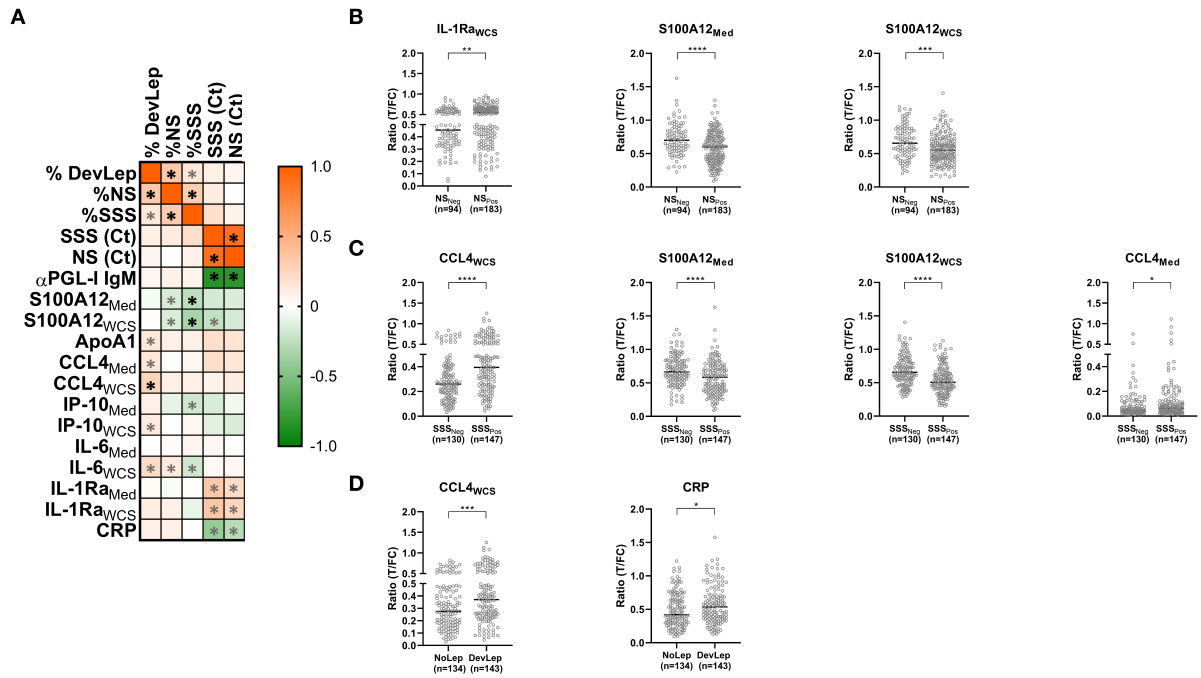

FIGURE 3 | Correlation of leprosy disease and M. leprae infection/colonization status in households with innate immune markers. **(A)** Whole blood without stimulus (Med) or stimulated with *M. leprae* whole cell sonicate (WCS) was frozen after 24 h. Levels of 8 proteins (αPGL-I IgM, S100A12, ApoA1, CCL4, IP-10, IL-6, IL-1Ra, and CRP) were assessed by up-converting phosphor lateral flow assays (UCP-LFAs) in supernatants of WBA for 31 households of index cases with multibacillary (MB) leprosy (bacterial index ≥2). The proportion of household contacts (HCs) diagnosed with leprosy upon first clinical examination (%DevLep) or with *M. leprae* DNA presence in nasal swabs (%NS) or slit-skin smears (%SSS) was calculated per household. These percentages and the RLEP Ct values determined by qPCR in NS and SSS were correlated with the levels of the assessed immune markers. The heatmap indicates the correlation coefficient (R), ranging from −1 (green) to 1 (orange) as determined using GraphPad Prism. Significant correlations ($p < 0.05$) are indicated with an asterisk (*), highly significant ($p < 0.0001$) are indicated with a black asterisk (*). **(B)** Significantly different ($p < 0.05$) levels of immune markers observed in HCs of *M. leprae* DNA positive (NS_{Pos}) and negative (NS_{Neg}) households. Ratio values (*y*-axis) represent the level of the assessed marker and were determined by dividing the signal of the test line (T) by the signal of the flow control (FC) line of the up-converting phosphor lateral flow assays. **(C)** Significantly different ($p < 0.05$) levels of immune markers observed in HCs of *M. leprae* DNA positive (SSS_{Pos}) and negative (SSS_{Neg}) households. **(D)** Significantly different ($p < 0.05$) levels of immune markers between HCs living in households where leprosy was diagnosed among contacts (DevLep) and in households where leprosy was not observed (NoLep).

Characteristics of the index case, such as the amount of *M. leprae* bacilli in NS or the αPGL-I antibody titer, had little influence on the development of leprosy nor on *M. leprae* colonization/infection in other household members. Physical distance of HCs to the index case was, however, significantly correlated with *M. leprae* colonization, though not with *M. leprae* infection or development of leprosy demonstrating the role of innate immune responses to remove bacteria.

In this study, S100A12 was associated with a protective response to *M. leprae* colonization/infection in HCs. As previously demonstrated (19), S100A12 also remained a useful marker to discriminate leprosy patients from EC. S100A12 has a dual role inducing both proinflammatory and antimicrobial effects by interacting with different receptors, such as RAGE and TLR4 (28). RAGE expression is associated with disease severity and levels of proinflammatory cytokines in active tuberculosis (TB) (29). Contrary, RAGE is protective against the development of pulmonary TB in mouse models (30) in line with reduction of antimicrobial activity in human macrophages upon TLR2/1 ligand activation by S100A12 knockdown (20). S100A12 thus seems to protect exposed individuals from *M. leprae* colonization and infection, but once infected, S100A12 can contribute to maintain a detrimental, pro-inflammatory state in leprosy patients.

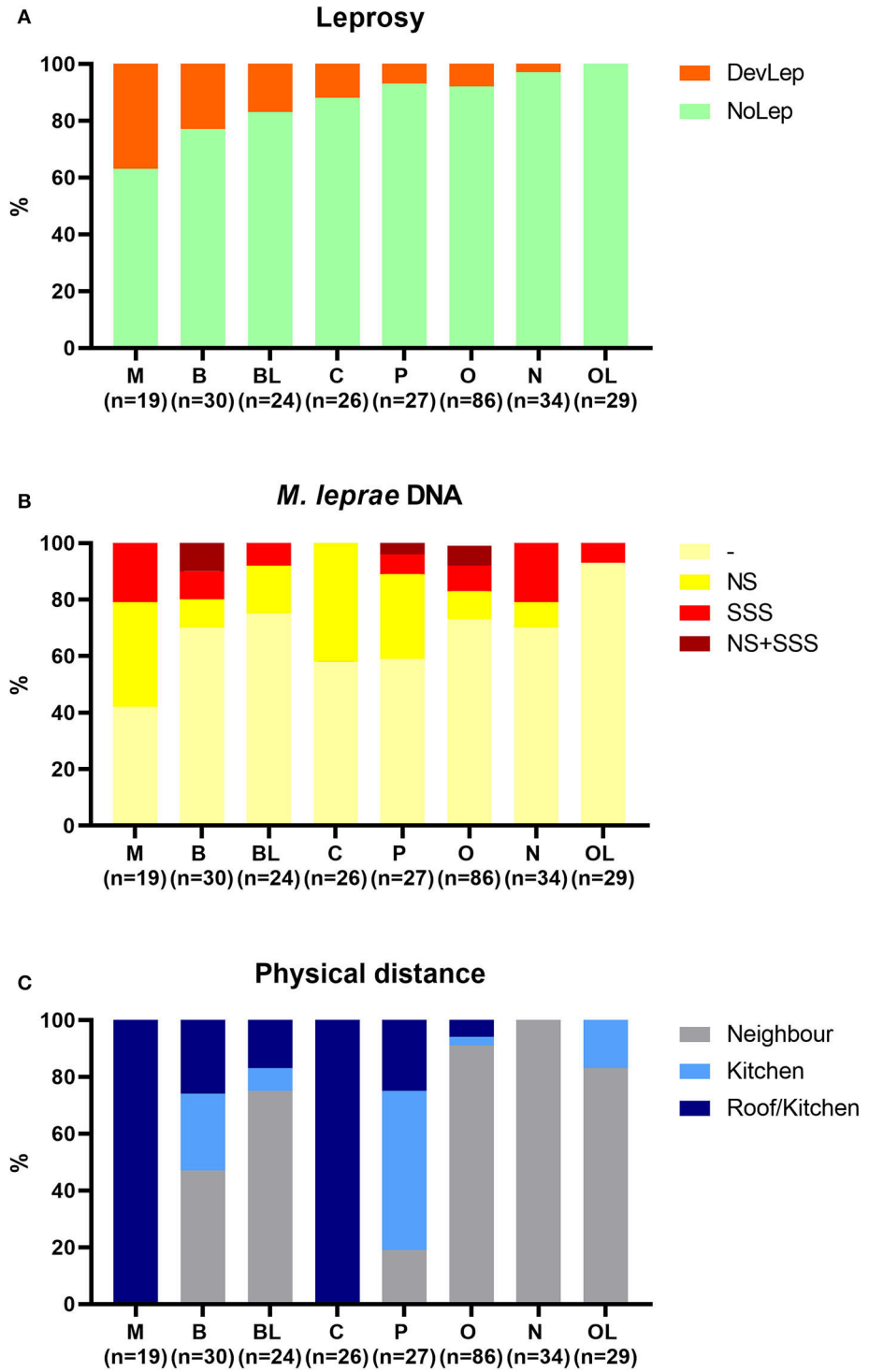

FIGURE 4 | Stratification of household contacts by genetic distance to the index case. Eight different groups were classified for genetic distance: spouse (M), child (C), parent (P), sibling (B), other relative (O), brother/sister in law (BL), other relatives in law (OL), and not family related (N). **(A)** Percentage of individuals diagnosed with leprosy upon first clinical examination (DevLep; orange) stratified by genetic distance and ranked by percentage. **(B)** Percentage of *M. leprae* DNA presence in nasal swabs (NS; yellow), slit-skin smears (SSS; red) or both (NS + SSS; dark red) stratified by genetic distance. **(C)** Distribution of physical distance (Roof/kitchen = dark blue, kitchen = blue, Neighbor = gray) to the index case stratified by genetic distance.

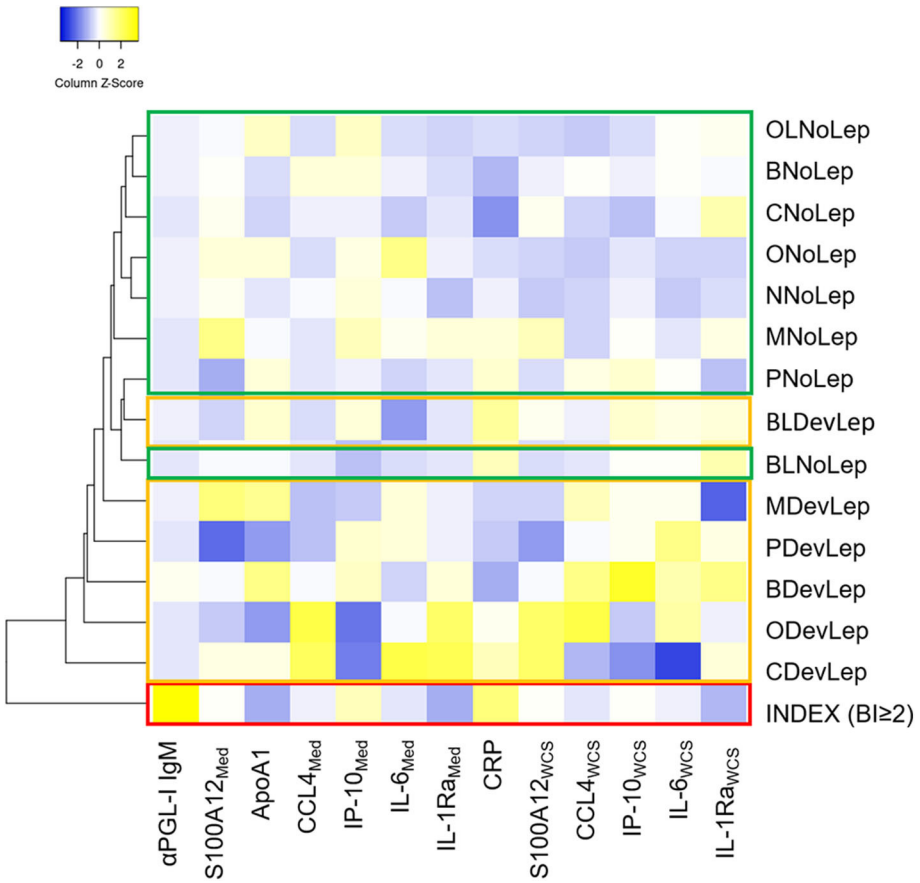

FIGURE 5 | Contacts diagnosed with leprosy upon first clinical screening cluster together based on their immune response, irrespective of genetic distance. Whole blood without stimulus (=Med) or stimulated with *M. leprae* whole cell sonicate (=WCS) was frozen after 24 h. Levels of 8 proteins (αPGL-I IgM, S100A12, ApoA1, CCL4, IP-10, IL-6, IL-1Ra, and CRP) were assessed by up-converting phosphor lateral flow assays in supernatants of whole blood assays (WBA) for 31 households of index cases with multibacillary (MB) leprosy (bacteriological index ≥2). The heatmap shows clustering based on average linkage performed by heatmapper (27) of the median level of eight serum protein markers in contacts diagnosed with leprosy upon first clinical screening of the HCs (DevLep) and without leprosy (NoLep) stratified by genetic distance; spouse (M), child (C); parent (P); sibling (B); other relative (O); brother/sister in law (BL); other relatives in law (OL) and not family related (N). The z-score indicates the deviation from the average level of the marker across groups, higher Z-scores are indicated in yellow and lower Z-scores in blue. Red = index case, yellow = contacts diagnosed with leprosy; green = household contacts without leprosy.

ApoA1 levels in HCs were similar to those in PB patients, suggesting that ApoA1 plays a role in limiting bacterial growth. This is in line with the finding that PB patients showed a similar low rate of *M. leprae* DNA presence in NS and SSS as HCs (25). Increased levels of ApoA1 have been observed in cells exposed to activated complement, where ApoA1 inhibits the formation of the membrane attack complex thereby contributing to complement clearance (31). Decreased levels are associated with destructive chronic inflammation, as ApoA1 exerts anti-inflammatory effects (32). The effects of ApoA1 do, however, not only rely on the protein level but also on the functionality, oxidative modification can for instance transform ApoA1 to an inflammatory agent (33). The role and functionality of ApoA1 in leprosy thus remains to be further elucidated. The influence of ApoA1 on lipid metabolism is of interest as dysfunctional high-density lipoprotein (involved in cholesterol transport to the liver of which the main protein is ApoA1) related to altered ApoA1 levels has been observed in MB patients (34). Moreover, it was suggested that *M. leprae* can directly affect ApoA1 biosynthesis.

Other markers in this study were associated with *M. leprae* colonization (IL-1Ra), whereas CCL4 was associated with infection and disease. These responses were most profound upon stimulation with *M. leprae* WCS, reflecting the innate immune response of these individuals to mycobacterial antigens. Interestingly, in whole blood of BCG-vaccinated infants the production of IL-1Ra and CCL4 was decreased upon stimulation of several TLRs (35). This observed response can be a result of BCG-induced trained innate immunity, which is immunological memory of the innate immune response that leads to an enhanced response to a subsequent trigger (36). Moreover, in Systemic Lupus Erythematosus (SLE) a pathogenic three-marker signature, including high levels of IL-1Ra and CCL4, was identified in monocytes (37). The signature was associated with the immune dysregulation in this autoimmune disease, in which

flares occur similar to leprosy reactions (38). High levels of IL-1Ra and CCL4 thus seem indicative of pathogenic innate immune responses, corroborating earlier results on the identification of IL-1Ra and CCL4 as biomarkers associated with a pathogenic immune response to *M. leprae* (18, 19, 39).

One of the challenges of application of host immune markers for diagnostics is the influence of co-morbidities or co-infections on biomarker levels. Helminth infections dampen the Th1 response and increase the risk for MB leprosy (40, 41). A biomarker study to examine the influence of helminth co-infection in leprosy patients is currently ongoing. Moreover, the influence on biomarker levels of co-morbidities, such as diabetes mellitus which is known to increase the risk of active TB (42), on the disease outcome should be further studied. Another issue impeding straightforward implementation of biomarkers is that inflammatory markers are not disease-specific. For example, S100A12 has been described as biomarker for rheumatoid arthritis (43), TB (44) as well as inflammatory bowel disease (45). As the UCP-LFA allows quantitative measurement of biomarkers it would be interesting to compare disease-specific S100A12 levels for these conditions. Taking into account the multiple factors that influence host immune responses, a biomarker signature that combines several innate immune markers is required to identify individuals at risk of developing leprosy. This signature should also be evaluated in other inflammatory conditions.

In conclusion: Frequent exposure of HCs to *M. leprae* results in a continuously active innate immune response. This allows differentiation of HCs from EC by user-friendly diagnostic tests measuring specific serum protein levels. If the innate immune response is sufficient, pathogens, and pathogen-infected cells are being successfully removed. However, prolonged (intense) activation can lead to an immune response directed against the host (46). The resemblance of the innate immune response of PB patients and HCs observed in this and previous studies (19, 39) indicates that PB leprosy can be a result of an imbalance in innate immunity. HCs that do not develop disease seem to effectively clear the bacteria without overactivation of the innate immune response. Elucidation of this delicate balance in innate immune responses by quantitation of appropriate biomarker signatures (47) can contribute to the identification of individuals at risk of developing leprosy upon *M. leprae* exposure. To gain more insight in this balance longitudinal analysis is required, which is currently ongoing. Diagnostic user-friendly rapid tests, as applied in this study, that allow quantitative measurement of combinations of innate immune markers represent useful tools to identify individuals that could benefit from prophylactic treatment.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local ethical board in Bangladesh (BMRC/NREC/2010-2013/1534). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AG, AH, and JR: designed research. AS, KA, and MK: enrolled patients, performed, and registered clinical diagnosis. AH, MT-C, EV, DJ, ET, and MK: performed experiments. PC and KA: resources. AH, MT-C, and AG: analyzed the data. AH and AG: wrote the paper. All authors: critically reviewed and agreed with the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01811/full#supplementary-material

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frontiers in Immunology

REVIEW
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Basophils and Eosinophils in Nematode Infections

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Helminths remain one of the most prolific pathogens in the world. Following infection helminths interact with various epithelial cell surfaces, including skin, lung, and gut. Recent works have shown that epithelial cells produce a series of cytokines such as TSLP, IL-33, and IL-25 that lead to the induction of innate and acquired type 2 immune responses, which we named Type 2 epithelial cytokines. Although basophils and eosinophils are relatively rare granulocytes under normal conditions (0.5% and 5% in peripheral blood, respectively), both are found with increased frequency in type 2 immunity, including allergy and helminth infections. Recent reports showed that basophils and eosinophils not only express effector functions in type 2 immune reactions, but also manipulate the response toward helminths. Furthermore, basophils and eosinophils play non-redundant roles in distinct responses against various nematodes, providing the potential to intervene at different stages of nematode infection. These findings would be helpful to establish vaccination or therapeutic drugs against nematode infections.

Keywords: helminth, nematode, allergy, basophil, eosinophil, Th2, type 2 immunity, Type 2 epithelial cytokines

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INTRODUCTION

Basophils and eosinophils, first described by Paul Ehlrich in 1879, are granulocytes (1, 2). Basophils and eosinophils are relatively rare when compared to other leukocytes. Basophils and eosinophils predominantly exist at most 0.5% and 5%, respectively, in peripheral blood under normal conditions, and have short half-lives when compared to lymphocytes. Intriguingly, basophils and eosinophils are evolutionally conserved in many animal species, suggesting their crucial and beneficial roles *in vivo*.

Basophils share some features with tissue-resident mast cells, which are abundant in peripheral tissues and long-lived cells. Basophils and mast cells are characterized by the expression of basophilic granules, surface expression of FcεRI, a high affinity IgE receptor, and release of chemical mediators (i.e., histamine) in response to cross-linking of surface IgE binding to FcεRI by antigens. Eosinophils have eosinophilic granules that contain eosinophil peroxidase (EPO), major basic protein, ribonulease cationic protein and eosinophil-derived neurotoxin, which are associated with allergic disorders and protection against parasites. In addition, Interleukin-5 receptor subunit-α on eosinophils define their unique biology in response to IL-5 produced by ILC2 and memory Th2 cells. Despite rarities of basophils and eosinophils in homeostatic conditions, basophils and eosinophils are found with increased their frequencies in peripheral tissues and play nonredundant roles in type 2 immune responses such as allergic inflammation and helminth infections. In the past 15 years, several works using deficient mice and specific-Cre mice

for basophils or eosinophils characterized the indispensable roles of basophils and eosinophils in pathophisiology. In this review, we summarize the latest research on the pivotal and nonredundant roles of basophils and eosinophils in nematode infection. This review would be helpful to establish vaccination or therapeutic drugs against nematode infections.

DEVELOPMENT OF BASOPHILS AND EOSINOPHILS

Granulocytes develop from pluripotent $CD34^+$ granulocyte progenitor (GP) cells in bone marrow through granulocyte/monocye progenitors (GMPs). GMPs derive the eosinophil lineage-committed progenitors (EoPs), and pre-basophil and mast cell progenitors (pre-BMPs) in bone marrow and the basophil/mast cell progenitors (BMCPs) in spleen. The pre-BMPs and BMCPs give rise to the mast cell progenitors (MCPs) and the basophil progenitors (BaPs) (3, 4) Controversially, Mukai et al. mentioned that BMCPs developed into mast cells, and not into basophils (5). The differentiation of basophils is regulated by Signal transducer and activator of transcription 5 (STAT5), the transcription factor distal promoter-derived Runt-related transcription factor 1 (Runx1), Interferon Regulatory factor 8 (IRF8), GATA binding protein 1 (GATA-1), GATA-2, and CCAAT/Enhancer Binding Protein α (C/EBPα) (5–10). STAT5 signaling is required for the differentiation of pre-BMPs into both basophils and mast cells through induction of GATA2, C/EBPα, and Microphthalmia-Associated Transcription Factor (MITF) that is important for differentiation of mast cells. Runx1-deficient mice exhibit a reduction of BaPs and basophils. Expression of IRF8 in GPs that assumingly develop from GMP to give rise to pre-BMP and BMCPs, is important for development of basophils upstream of GATA-2. The differentiation of eosinophils is regulated by GATA binding protein 1 (GATA-1), PU.1, and the CCAAT-enhancing binding protein (c/EBP) family of transcription factors. GATA-1 and PU.1 synergistically promote transcription of major basic protein (MBP). The absence of both MBP and EPO resulted in near complete loss of eosinophils *in vivo* (11).

GATA-1 reprograms immature myeloid cells to develop three different hematopoietic progenitor lineages: erythroid cells, megakaryocytes and granulocytes. GATA-1 is essential for maturation of erythroid and megakaryocyte precursors and positive autoregulation of GATA-1 expression is mediated by high affinity palindromic GATA-binding sites in the GATA-1 promoter (12, 13). Deletion of these GATA-binding sites in mice (called ΔdblGATA mice) results in a complete ablation of mature eosinophils (14). ΔdblGATA mice exhibit normal platelet development, and red blood cell production is only subtly impaired, but GATA-1 null mice have an embryonic lethal phenotype, with profound anemia and defective megakaryocyte development. As a result of these findings, ΔdblGATA mice were used as model of "eosinophil-deficient" mice, but later studies have defined additional roles for GATA-1 in the development of basophils and mast cells (15). GATA-1 expression is involved in the development and activity of megakaryocyte/erythrocyte progenitors, basophil/mast cell progenitors, basophil progenitors, mast cell progenitors and eosinophil progenitors but not granulocyte/monocyte progenitors (16–19). More recent studies have shown that ΔdblGATA mice exhibit additional defects in the generation of basophil precursor cells (BaP) and mature basophils (3, 20). Furthermore, basophils that do develop in ΔdblGATA mice have impaired IL-4 production and CD63 expression after cross-linking of antigen-specific IgE. Knockdown of GATA-1 in basophils *in vitro* resulted in defective basophil development, reduced degranulation and lower production of IL-4 in response to antigen stimulation. These suggested that defects in basophils of ΔdblGATA mice are due to decreased expression of GATA-1. In contrast to basophils, mast cell development in ΔdblGATA mice is not overtly impacted (21, 22). Similar to this, GATA-1-deletion does not affect development of mast cells *in vivo* and *in vitro* (23, 24). Collectively, ΔdblGATA mice showed developmental and functional impairments in basophils and eosinophils. In addition, the transcription factor GATA-1 controls both basophils and eosinophils.

BASOPHILS

Basophilia in Parasite Infection

Although basophils make up a small proportion (<0.5%) of leukocytes in the blood, they accumulate in peripheral tissues during type 2 inflammation. Infiltration of basophils is observed in local lesions after helminth infection, and allergic skin diseases, implying that they may play important roles in supporting the inflammation (25, 26). Similar to allergic diseases, basophils accumulate in skin lesions of humans and mice after infestation with ectoparasites (27–29). However, unlike mice, blood basophilia rarely occurs in humans following nematode infections (30).

$CD4^+$ T cell-derived IL-3 is critical for the survival and proliferation of basophils during a nematode infection (31). IL-3 activates basophils to produce IL-4 through IL-3Rα chain and FcRγ chain complex (32). Thymic stromal lymphopoietin (TSLP) induced by helminth infection, supports basophil proliferation and promotes induction of Th2 cytokine responses in *Trichinella* infection (33). During *Heligmosomoides polygyrus* (Hp) infection, IL-3, IgG1, and IgE selectively promote basophil expansion (34). IgE signaling promotes IL-3Rα chain expression on basophils (35). The factors that drive basophil expansion downstream of the IgE/FcεRI axis are still unknown. In mast cells, IgE induces survival *via* binding to FcεRI on mast cells by signaling through Bfl-1, a Bcl-2 family protein. However, the IgE/FcεRI/Bfl-1 axis apparently is not operative in human basophils (36, 37).

Basophils and Type 2 Epithelial Cytokines

TSLP, IL-33, and IL-25 are predominantly produced from barrier epithelial cells to initiate type 2 immune responses, including eosinophilia. Thus, they are referred to as Type 2 epithelial cytokines.

Basophils express receptors for TSLP and IL-33 (38). TSLP activates basophils to produce IL-4, resulted in establishment of Th2 cell-dependent immunity (38). IL-33 activates basophils and mast cells to enhance the degranulation and production of cytokines such as IL-4, IL-6, and IL-13 (39). IL-33-mediated basophil activation has been discussed in atopic dermatitis (40). Single Nucleotide Polymorphisms (SNPs) in both *TSLP* and *CRLF2* coding TSLP receptor result in increased expression or signaling, and have been associated with Eosinophilic esophagitis (EoE) (41). In addition, IL-33 cytokine and receptor (*IL1RL1*, ST2) signaling is elevated in gastrointestinal allergic diseases, including food allergy and EoE (42). Of note, ST2 expression on basophils is necessary for basophil accumulation in the esophagus and the development of experimental EoE. Basophils are also required for TSLP-mediated EoE and IL-33-mediated food allergy in mice (43, 44).

The role of basophils in TSLP-dependent inflammation has been studied well, using topical vitamin D3 analog (MC903)-induced model of atopic dermatitis. Basophil-specific IL-4-deficient mice (IL-4 3'UTR mice) exhibit impaired ear swelling, reduced levels of antigen-specific serum IgE and diminished production of type 2 cytokines in lymph nodes after topical MC903 treatment (21). In addition, TSLP-stimulated basophils enhance ILC2 responses through production of IL-4, resulting in skin inflammation (45). However, basophil-specific TSLPR-deficiency by bone marrow chimerism does not confer protection from cutaneous inflammation or limit serum IgE titers after topical MC903 treatment (46). Furthermore, Basophil-specific TSLPR-deficiency in $Mcpt8^{cre}Tslpr^{fl/fl}$ mice, did not impair the severity of the airway inflammation, generation of Th2 cells or levels of serum IgE when compared to control mice after intranasal challenges of antigen with MC903, suggesting that this type 2 inflammatory response was mediated by TSLPR on DC and $CD4^+$ T cells, but not basophils and ILC2 cells (47).

Basophils and Th2 Differentiation in Helminth Infection

Basophils promote Th2 cell differentiation through IL-4 production during *Trichinella spiraris* (Ts), *Heligmosomoides polygyrus* (Hp) and *Litomosomoides sigmodontis* Filaria infections (33, 48, 49). Giacomin et al. showed that deficiency of TSLPR, but not IL-3R, impaired basophilia in draining lymph nodes during Ts infection, which is associated with reduction of Th2 cells. Also, Th2 cell-mediated immune responses are important for expulsion of Hp parasites during re-infection (50).

Naïve $CD4^+$ T cells require the interaction with peptide-loaded MHC class II (MHC-II) complexes on antigen presenting cells (APCs) to differentiate into Th2 cells, so Th2-differentiation could be primed by basophils. Pioneering work by Hida et al. showed that basophils produce IL-4 to support APCs, and promote Th2 cell differentiation (51). This finding was supported by follow-up studies from other research groups (52, 53). Later, three independent groups observed that basophils express MHC-II and secrete IL-4 to induce the differentiation of naïve $CD4^+$ T cells to Th2 cells. Furthermore, depletion of basophils by anti-FcεRIα antibody (clone MAR-1) diminished Th2 cell differentiation *in vivo*. These findings suggest that basophils are professional APCs that express peptide-loaded MHC-II, induce Th2 differentiation in a cysteine protease papain-administration model, IgE and antigen-induced model and *Trichuris muris* (Tm) in primary infection (54–56). Yoshimoto et al. also showed that human basophils express MHC-II in that paper. However, the role of basophils as APCs is still under discussion. (1) Although all three papers used anti-FcεRIα antibody MAR-1 antibodies to deplete basophils *in vivo*, recent work revealed that MAR-1 binding is not limited to FcεRIα, but this antibody can also non-specifically bind to FcγRI and FcγRIV (57). Furthermore, treatment of MAR-1 depletes $CD11c^+$ inflammatory DC *in vivo* (57–59). (2) When compared to DCs and B cells, basophils express low levels of surface MHC-II. Basophils also do not express the proteins that are required for MHC-II-restricted antigen processing or presentation, including cathepsin S, H-2M and the invariant chain Ii, and they exhibit a minimal capacity to process and present antigen when compared with DCs (52, 53, 58, 59). Miyake et al. demonstrated that basophils acquire peptide-MHC-II complexes from DCs *via* trogocytosis to prime Th2 cells in MC903 treatment–induced atopic dermatitis model (60). To resolve these remaining discrepancies, further studies will be needed to compare MHC-II functions on DCs and basophils with CD11c-Cre and basophil-specific Cre mice, respectively. Although some of basophil-specific Cre was based on a gene of mMCP-8, the technical caveat is that mMCP-8 expression is not restricted to basophils (61, 62).

Basophils are important for the expulsion of Tm from the cecum in primary infection. In the Tm model of helminth infection, Webb LM et al. showed that basophil localization to the caecum, but not the spleen, is regulated by Notch expression (63). Thus, Notch signaling in basophils is critical to induction of type 2 immune responses, including Th2 cell generation, RELM-β expression in cecum and resulting Tm clearance.

Recently, it has been reported that basophils can inhibit the type 2 immune responses *via* increase of expression of neuromedin B (NMB) receptors on ILC2 cells in primary infection of Nb, suggesting that basophils are not always inducer or enhancer of Type 2 immune responses (64).

Basophils and M2-Type Macrophages in Helminth Infection

M2-type macrophages play key roles in regulating allergy and confer protective roles against helminths (50, 65–67). M2-type macrophages also protect against fatal lung damage in primary Nb infection (68).

Basophil depletion by Thy1.2 or CD200R3 antibody correlates with increased worm burden after re-infection with *Nippostrongylus brasiliensis* (Nb) (69–71) (**Figure 1** and **Table 1**), and this protection is mediated by basophil-derived type 2 cytokines (72). During Nb infection, cross-linking of Nb antigen-specific IgE promotes basophil activation and IL-4 production. Basophil-derived IL-4 promotes M2-type macrophage differentiation, and the production of anti-parasitic enzyme Arginase-1 to protect against

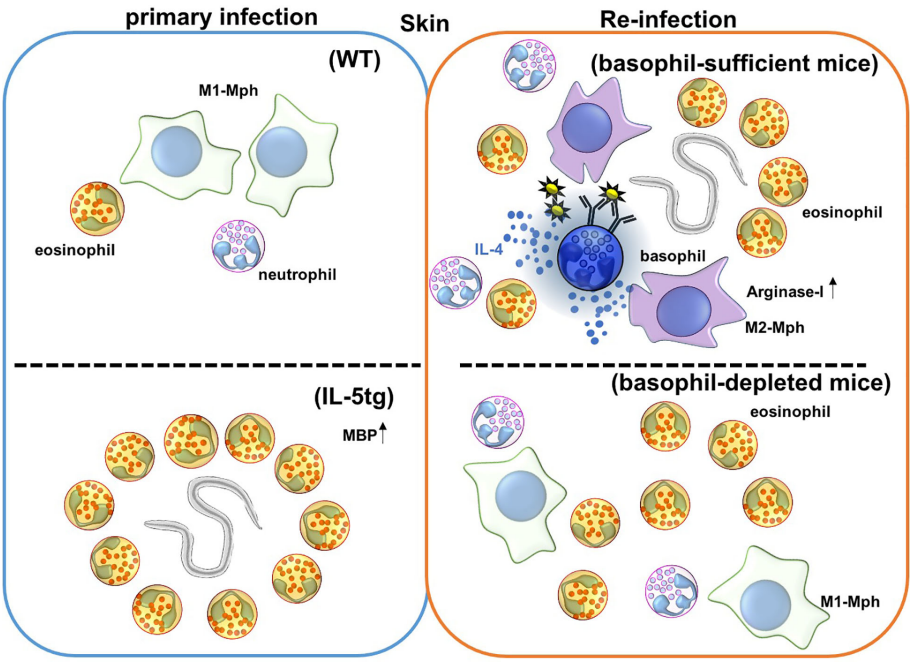

FIGURE 1 | Immune responses against *Nippostrongylus brasiliensis* (Nb) infection in the skin. Nb leaves the skin and enters the blood and lymphatic vessels within 20 minutes of primary infection. In IL-5 transgenic mice, eosinophils are activated by overexpression of IL-5, and produce the anti-helminthic enzyme, major basic protein (MBP), causing Nb parasites to arrest in the skin lesion during primary infection. However, Nb antigens activate basophils during secondary infection, and correspondingly activate monocytes/macrophages to promote M2-type macrophage differentiation. Activated M2-type macrophages then secrete the anti-nematode enzyme, Arginase-I. Eosinophils are also accumulated in the skin, but the role of these accumulated eosinophils is not yet known. Depletion of basophils allows for Nb parasites to leave the skin, but eosinophils still infiltrate the tissue during secondary infection.

secondary Nb infection in the skin (73). Similar to this, Depletion of macrophages expressing *Relma* that is a marker of M2-type macrophages increased worm burden in the lungs and gut (68). Conversely, basophil-deficiency did not affect the protection against a secondary subcutaneous inoculation of *Strongyloides venezuelensis* (Sv) or *Strongyloides ratti* (Sr), which originally penetrate skin to infect the hosts by a mechanism that is similar to Nb (74–76) (**Table 2**).

TABLE 1 | The experimental models of nematode infections.

Helminth	Infection stage	Natural Route of infection	Route of Experimental inoculation	Characteristics of Infection	Model for infection in human (infection route)
Nippostrongylus brasiliensis	L3 larvae	Skin penetration	i.d., s.c.	Naturally penetrate the skin and migrate to the lungs. Parasites then migrate to the gut to lay eggs. Short-lived infection	*Ascaris lumbricoides* (Oral ingestion) Hookworms; *Necator americanus* (Oral ingestion), *Ancylostoma duodenale* (Oral ingestion or skin penetration) Strongyloides stercolaris (Skin penetration)
Strongyloides venezuelensis *Strongyloides ratti*	L3 larvae	Skin penetration	s.c.		
Heligmosomoides polygyrus	L3 larvae	Oral ingestion	p.o.	Chronic infection from ingestion of larvae	
Trichinella spiralis	L1 larvae	Oral ingestion	p.o.	Food-borne (infective juvenile), zoonotic parasite	*Trichinella spiralis* (Oral ingestion)
Trichuris muris	Eggs	Oral ingestion	p.o.	Ingestion of infectious eggs that hatch in the cecum and colon	*Trichuris trichiura* (Oral ingestion)
Litomosoides sigmodontis	L3 larvae	mite	s.c., mite	Chronic infection	Human filarial diseases; *Brugia malayi*, *Brugia timori*, *Wuchereria bancrofti* *Onchocerca volvulus*, *Loa loa*
Brugia pahangi	L3 larvae	mosquito	s.c.	Adult worms inhabit the pleural cavity	

M2-type macrophages also provide protection against secondary infection of *Heligmosomoides polygyrus* (Hp), but M2-type macrophage differentiation during Hp infection is induced by IL-4 from $CD4^+$ T cells in small intestine (50). However, the expression of FcR, IL-4 and IL-13 on basophils is required for Th2 cell priming, downstream M2-type macrophage differentiation and Hp worm clearance (48). Taken together, after surface-bound IgE is cross-linked by helminth-derived antigens, basophils produce IL-4 and IL-13 to induce M2-type macrophage differentiation, resulting in expulsion of Nb and Hp from the skin and small intestine.

Non-basophils can also produce type 2 cytokines to induce M2-type macrophages in protection against Nb re-infection in the lungs (67, 82). As described by Chen et al. neutrophils in Nb-infected mice upregulated IL-13 transcripts in secondary infection, suggesting that neutrophils could promote M2-type macrophage activation in the lungs to clear Nb parasites (67). Conversely, another study showed that ILC2 and Th2 cells, but not neutrophils, could potentially induce M2-type macrophage activation to kill Nb at the lung during secondary infection (82).

Basophil-Derived Proteases

Basophil-derived proteases, including serine protease mouse mast cell protease-8 (mMCP-8), and tryptase mMCP-11, play an important role in promoting skin inflammation (83, 84). The mMCP-11 increases vascular permeability, allowing for increased migration of basophils, eosiniophils, macrophages and neutrophils. Intriguingly, mMCP-11-deficiency ameliorates IgE-mediated chronic allergic inflammation in the skin. Furthermore, intradermal administration of mMCP-8, induces the production of Cxcl1, Ccl2, and Ccl24, which recruit neutrophils, monocytes, and eosinophils into the lesion. Similar to atopic dermatitis, Nb re-infection is characterized by increased numbers of neutrophils, macrophages, eosinophils and basophils in the skin lesion and high titers of IgE in the serum. However, the role of these proteases in anti-helminth immunity is not yet known.

Basophils are involved in resistance against both *Strongyloides venezuelensis* (Sv) and *Strongyloides ratti* (Sr) in primary infection (75, 76). The contributions of basophils in induction and expansion of Th2 cells are negligible, although those parasites are expelled by type 2 immune responses from small intestine. It might be possible that basophil-specific molecules such as mMCP-8 and mMCP-11 are associated with the protection against these nematodes.

EOSINOPHIL

Eosinophils make up about 5% of leukocytes in peripheral blood, and have a short half-life in circulation. However, the number of circulating eosinophils are increased in patients with allergic diseases and helminth infections. Eosinophil granules contain major basic protein, eosinophil cationic protein, eosinophilic peroxidase and eosinophil-derived neurotoxin.

Approximately, 7–10% of the total protein content of human eosinophils consists of galectin-10, while mice do not contain a functional galectin-10 gene (85, 86). Upon Eosinophil activation, secreted galectin-10 protein forms aggregates, called Charcot-Leyden crystals, at sites of inflammation. Charcot-Leyden crystals were first described as extracellular bipyramidal crystals in the airways of patients with asthma in 1853 by Charcot, and this observation was confirmed by Leyden in 1872. However, the link between Charcot-Leyden crystals and eosinophilic airway disease and/or mucus production was largely forgotten for over 100 years. Recent work from Persson et al. showed that intratracheal administration of galectin-10 promoted the infiltration of neutrophils and monocytes, and Th2 cell priming (87).

GM-CSF, IL-3, and IL-5 accelerate the growth, maturation, survival, and activation of eosinophils. GM-CSF-deficient mice exhibit impaired recruitment of eosinophils to airways in a model of allergic airway. IL-5 deficiency is correlated with a 2- to 3-fold reduction in B-1 cells and eosinophils as compared control mice. However, the eosinophils that did develop in IL-5–deficient mice were morphologically similar to eosinophils in

TABLE 2 | The role of basophils and eosinophils in nematode infections.

Helminth	The role of Basophils	The role of eosinophils	Reference
Nippostrongylus brasiliensis	- Basophils protect from re-infection in the skin.	- $CXCR6^+ST2^+$ mTh2 cells facilitate eosinophilia in the lungs to reduce the fecundity in the lungs in re-infection.	(73)
Strongyloides venezuelensis	- Basophil-depletion in Mcpt8DTR mice revealed small contribution of basophils in primary infection and minor or no roles in secondary infection.	- The duration of Sv was increased in ΔdblGATA mice in primary infection (unpublished data)	(77) (75) (74)
Strongyloides ratti	- The number of intestinal nematodes and fecal eggs is elevated in Mcpt8-Cre mice.	- IL-5 deficiency increased the number of intestinal worms and fecal eggs.	(76) (78)
Heligmosomoides polygyrus	- Mcpt8-Cre mice have a high number of eggs in feces during re-infection.	- The fecundity of Hp was increased in ΔdblGATA and PHIL mice during re-infection.	(48) (79)
Trichinella spiralis	- Th2 immune response is reduced in Bas-TRECK mice.	- Eosinophils increased the survival of muscle larvae	(33) (80)
Trichuris muris	- Basophil depletion *via* MAR-1 treatment increases the number of Th2 cells and impairs Tm expulsion.	- Eosinophil depletion by anti-IL-5 Ab treatment does not change worm expulsion.	(54) (81)

control mice, but IL-5–deficient mice failed to develop blood and tissue eosinophilia in response to helminth infection (88). IL-5 transgenic (IL-5tg) mice overexpressing IL-5 in homeostatic condition have elevated numbers of circulating eosinophils, neutrophils, lymphocytes and monocytes (89).

Eosinophils and Type 2 Epithelial Cytokines

Type 2 epithelial cytokines activate ILC2 and Th2 cells to produce IL-5 and IL-13, leading to eosinophil infiltration in allergic inflammation and helminth infection (77, 90, 91).

It has been reported that eosinophils express own receptors for Type 2 epithelial cytokines. Human eosinophils express functional TSLP receptor components: TSLPR and IL-7Rα. TSLP up-regulates the expression of adhesion molecule CD18 and intercellular adhesion molecule-1, while down-regulating L-selectin, resulting in increased migration by eosinophils to promote tissue eosinophilia (92). TSLP also induces eosinophil degranulation and the release of eosinophil extracellular traps to capture extracellular bacteria (93). Although TSLP supports the survival of various leukocytes including T cells and non-hematopoietic cells, the role of TSLP in maintaining eosinophil survival is controversial (94–96). Two studies examined the role of TSLP in survival of eosinophils; while one reported to enhance survival of eosinophils, the other did not report a notable change in eosinophil survival (97, 98). These results suggest that eosinophils are involved in pathogen defense when TSLP production is triggered by environmental factors. Furthermore, tuft cells located in mucosal epithelial layer predominantly produce IL-25 to activate ILC2 cells (99). Tuft cells monitor the microbial metabolite succinate to initiate type 2 inflammation including tuft cell and goblet cell hyperplasia, and eosinophilia (100).

Eosinophil in the Skin in Helminth Infections

Eosinophilia in the skin occurs during re-infection with *Nippostrongylus brasiliensis* (Nb), while ΔdblGATA mice lacking eosinophils are susceptible to re-infection of Nb. Thus, eosinophils are believed to play an important role in providing protection during Nb re-infection (101, 102). As we mentioned above, since ΔdblGATA mice display numerical and functional aberrancy in basophils, adoptive transfer of wild-type basophils into ΔdblGATA mice confers the protective immunity against Nb in the skin in re-infection (20). Antibody-mediated depletion of eosinophils, with a combination of anti-IL-5 and anti-Siglec-F antibodies does not change the Nb parasite burden in the lungs, suggesting that basophils, rather than eosinophils, are primarily important for providing protection from Nb in skin in re-infection (77) (**Figure 2**). The role of eosinophils in the skin during Nb re-infection is not yet clear, but eosinophils have known roles in tissue repair and help the helminth infection. Eosinophils promote skin tissue repair by producing TGF-β during the resolving phase of inflammation (103). It has been also known that eosinophils promote *Trichinella spiralis* (Ts) infection; eosinophils help survival of Ts larva in the muscles of hosts (104). Eosinophils increase the fecundity of *Heligmosomoides polygyrus* (Hp), and reduce IL-4 response by follicular helper T cells and IgG1 class-switching in peyer's patches in re-infection (79).

Eosinophils in the Lungs in Helminth Infection

Eosinophils are recruited into Nb-infected lungs during primary and secondary infection by ILC2 and $CXCR6^+ST2^+$ memory Th2 cells (77, 82, 90, 105) (**Figure 1** and **Table 1**). ILC2 and $CXCR6^+ST2^+$ memory Th2 cells express IL-33 receptors and produce high concentrations of IL-5 and IL-13 during allergic responses and parasitic infections (91, 106, 107). Eosinophil-deficiency alone does not change the duration of primary Nb infection, but eosinophils are required to stall parasite maturation in the lung during re-infection with Nb (77, 108). Adoptive transfer of $CXCR6^+ST2^+$ memory Th2 cells from Nb-sensitized mice conferred resistance to Nb in the lungs of recipient mice. IL-5 is also required to induce major basic protein (MBP) secretion by eosinophils (77). Adoptive transfer of eosinophils, but not MBP-depleted eosinophils, into the lungs inhibited Nb maturation. MBP expression in eosinophils is also required for eosinophils to kill *Strongyloides stercoralis* (Ss) parasites in implanted cell-impermeable diffusion chambers (109). Collectively, these findings suggest that eosinophils protect from Nb re-infection in the lungs but not skin, and that MBP produced by eosinophils is required for protection against Nb and Ss.

IL-5 transgenic (IL-5tg) mice overexpressing IL-5 in homeostatic condition have elevated numbers of circulating eosinophils, neutrophils, lymphocytes and monocytes (89). Since IL-5tg mice exhibit a pronounced elevation of eosinophils, they are classically used to model chronic eosinophilia in mice. IL-5tg are strongly resistant to several helminth infections, including Nb and Ss (109, 110). Adoptive transfer of eosinophils from IL-5tg mice conferred the protection against Nb. These imply that high levels of IL-5 confer the capacity to protect from Nb infection to eosinophils (111). In the same context, Yasuda et al. demonstrated that Sv infection prior to Nb infection caused mice to acquire a highly responsive "trained" phenotype. This trained phenotype was associated with a reduction in the number of Nb larvae in the lungs as a result of an enhanced accumulation of ILC2 cells that produced IL-5 and IL-13 to promote pulmonary eosinophilia (107).

BASOPHIL, EOSINOPHILS, AND ANTIBODY PRODUCTION DURING HELMINTH INFECTION

B cells are required for protection from various infections of nematodes including *Strongyloides venezuelensis*, *Nippostrongylus brasiliensis* (Nb), *Trichuris muris* and *Heligmosomoides polygyrus* (48, 73, 74, 112), and both basophils and eosinophils are crucial for the production and maintenance of parasite-specific antibodies during *Trichinella spiraris* infection (49, 80). Early evidence suggests that basophils, $CD4^+$ T cells and B cells provide interconnecting roles in the response to parasitic infection,

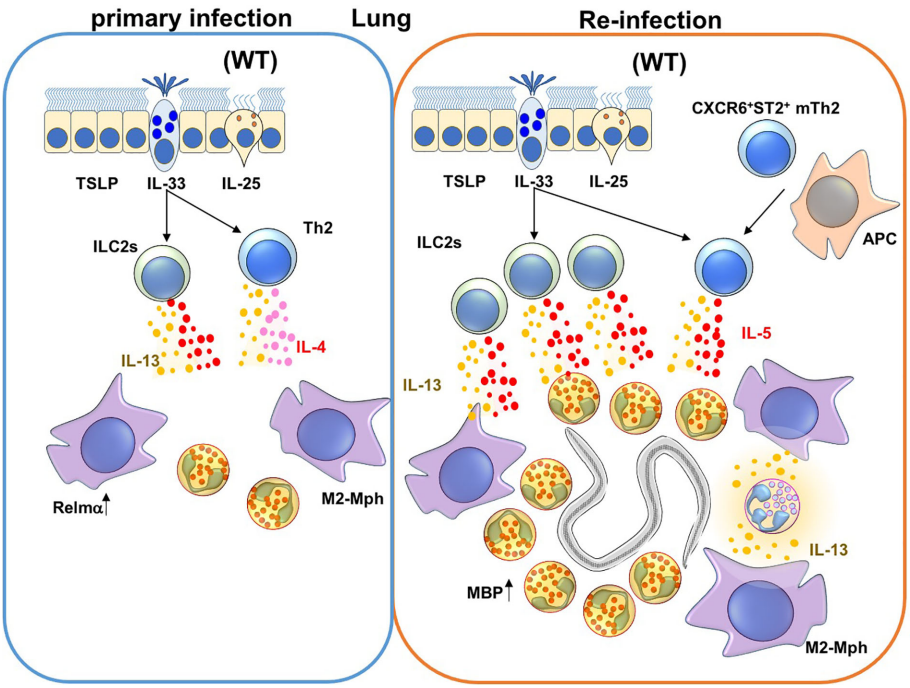

FIGURE 2 | Immune responses against *Nippostrongylus brasiliensis* (Nb) infection in the lungs. During a primary Nb infection, Nb leaves the lungs before the peak of infiltration of eosinophils and M2-type macrophages to the site of lung infection (68). On the other hand, during re-infection of Nb, activated Innate Lymphoid Cell 2 cells (ILC2s) and CXCR6$^+$ST2$^+$ memory Th2 cells produce IL-5 and IL-13. IL-5 and IL-13 promote lung infiltration, activation and major basic protein (MBP) production by eosinophils. In addition, M2-type macrophages are also induced by type 2 cytokines that could be produced by ILC2s, Th2 cells and/or neutrophils in re-infection.

but the mechanisms that coordinate these cells remain poorly understood.

Activated basophils express CD40 ligand and secrete IL-4 and IL-13, which are required for IgE class-switching and production during parasitic infection (113–116). However, this basophils activation is predominantly mediated by FcεRI cross-linking (117), suggesting that the capacity for basophils to activate B cell class-switch recombination occurs after the initial priming of parasite-specific B cells. Alternatively, after co-culture with basophils, CD4$^+$ T cells exhibit an augmented capacity to induce IgE class-switching in B cells (118), so basophils could also prime IgE responses through a CD4$^+$ T cell intermediate. After class-switching, basophils support the survival of plasma cells and memory B cells through the production of IL-4 and IL-6 in the spleen and bone marrow (118, 119), and eosinophils support the survival of plasma cells in the bone marrow through the secretion of IL-6 and APRIL (120).

IgE production relies on IL-4 production by follicular helper cells (Tfh) (121, 122). Tfh cells are divided into subsets by gene expression profiles and functional roles that mirror T-helper cell subsets in humans and mice; Tfh1, Tfh2, Tfh17, Tfh13, and follicular regulatory T (Tfr) cells (123–125). It has been reported that the generation of IL-4-expressing Tfh2 cells is facilitated by basophils in response to cross-linking of IgD on the basophil surface (126). More recently, Gowthaman et al. showed that a rare population of IL-13 producing Tfh (Tfh13) cells is required for the production of high affinity, anaphylactic IgE against allergens, whereas infection of Nb with OVA did not generate Tfh13 cells (125). However, Tfh13-induced IgE is regulated by Tfr cells in the germinal center, suggesting that Tfr cells could be limiting Tfh13 cells activity during Nb infection (127). The absence of Tfh13 in helminth infection could explain why high affinity IgE antibodies are not detected during helminth infection.

Collectively, basophils and eosinophils have the potential to contribute to the generation of IgE specific for helminth and helmith-derived antigens, and in turn, these antibodies coat FcεRI on basophils to arm them for rapid activation during re-infection of the helminth.

VACCINATION AGAINST HELMINTH

Litomosoides sigmodontis (Ls) is a filarial nematode parasite that is used as a model of filarial diseases. The parasites are inoculated or transmitted through the skin barrier by mites, and they inhabit the pleural cavity after developing into adult worms. When irradiated parasites are injected into mice as a method of vaccination, protective immunity against Ls larvae is induced in a basophil-dependent manner, but basophils are dispensable as effector cells against live Ls (128). Vaccination of mice with *Heligmosomoides polygylus* (Hp)-excretory secretory products confers the resistance against Hp larvae, but protective immunity depends on neutrophils, but not eosinophils, basophils or mast cells. However, basophils (but not eosinophils) do contribute to the

worm expulsion during secondary re-infection with Hp (48, 79, 129). Killing trapped parasites in the small intestine is partially dependent on eosinophils.

The adjuvant effects of TNF-α have been well documented. Mast cells produced TNF-α to orchestrate the recruitment of T cells and dendritic cells into draining lymph nodes in *Escherichia coli* or *Klebsiella pneumoniae* infections (130). Other studies showed that TNF-α and synthetic granules mimicking granules of mast cells can be used for vaccination (131). Recently, Piliponsky AM et al. published that basophil-derived TNF-α enhanced survival in a sepsis in mice (132). Together with this, it could be possible to use basophils as one of the primary targets for vaccination as the adjuvant function.

CONCLUSION

Here, we summarized the roles of basophils and eosinophils in nematode infections. We also showed that granulocytes are stringently controlled by type 2 epithelial cytokines, and control type 2 immune responses by promoting Th2 cell differentiation and antibody production. Recent findings demonstrate that basophils and eosinophils are key players in protective immune responses against helminths. Although basophils and eosinophils are not primarily associated with directly killing nematodes during primary infection, these cells hinder parasite burden during reinfection by enacting the rapid deployment of type 2 immune responses. In response to nematode parasite re-infection, basophils are armed with IgE specific for nematodes or their products and accumulate in the peripheral tissues. After antigen stimulation, basophils secrete IL-4 to induce M2-type macrophages, and proteases to rapidly recruit monocytes, neutrophils, and eosinophils to the infection site. In response to IL-5, eosinophils are activated to a "trained" phenotype and produce major basic protein (MBP) to kill nematodes. On the other hand, eosinophils can also support nematode survival and tissue repair during the resolving phase. Further studies are necessary to fully characterize how basophils and eosinophils coordinate their cell-specific responses to expel nematodes. However, therapeutic targeting of basophils and eosinophils or their products could be crucial for developing novel therapeutic interventions against nematode infections.

AUTHOR CONTRIBUTIONS

KO-N, PPD and SFZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Hepatitis B Virus Infection Among Leprosy Patients: A Case for Polymorphisms Compromising Activation of the Lectin Pathway and Complement Receptors

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Thousands of leprosy patients not only suffer from physical deformities, but also either have or have had hepatitis B virus (HBV) coinfection. Polymorphisms of the complement system modulate susceptibility to leprosy, but genetic susceptibility to past or present HBV infection is unknown. We used sequencing and multiplex sequence-specific PCR to genotype 72 polymorphisms of seven genes (*MBL2*, *FCN1, FCN2, FCN3, MASP1, MASP2, C3*) encoding components of the lectin pathway, and two genes encoding complement receptors (*CR1, VSIG4*) in 190 patients, of which 74 were positive for HBsAg and/or anti-HBc (HBV+, 93.2% with a resolved infection) and 116 lepromatous patients, and 408 HBV-blood donors. In addition, we tested for levels of proteins of the lectin pathway. We found no difference between serum concentrations of mannan-binding lectin (MBL), MBL-associated serine proteins (MASP-1, MASP-2, MASP-3, MAp44), ficolin-3 (FCN-3), soluble complement receptor 1 (sCR1) and MBL mediated C4 activation, measured by ELISA or TRIFMA in up to 167 HBV+ and HBV– patients. Haplotypes lowering protein levels or encoding dysfunctional proteins increased susceptibility to HBV infection: *MBL2*LYQC* (OR = 3.4, p = 0.02), *MASP1*AC_CC* (OR = 4.0, p = 0.015) and *MASP2*1C2-I* (OR = 5.4, p = 0.03). Conversely, *FCN1*3C2* haplotype, associated with higher gene expression, was protective (OR = 0.56, P = 0.033). Other haplotypes associated with HBV susceptibility were: *MASP2*2B1-i* (OR = 19.25, P = 0.003), *CR1*3A* (OR = 2.65, P = 0.011) and *VSIG4*TGGRCG* (OR = 12.55, P = 0.014). Some polymorphisms in ficolin genes associated with lower protein levels increased susceptibility to leprosy/HBV infection: *FCN*1* (OR = 1.66, P = 0.029),

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*FCN2*GGGCAC* (OR = 6.73, P = 0.008), and *FCN3*del_del_C* (OR = 12.54, P = 0.037), and to lepromatous disease/HBV infection: *FCN2*TA* (OR = 2.5, P = 0.009), whereas *FCN2*MAG* was associated with increased FCN-2 expression and resistance against coinfection (OR = 0.29, P = 0.026). These associations were independent of demographic factors and did not increase susceptibility to leprosy *per se*, except *MASP2*1C2-I*. Associations for *FCN2, FCN3, MASP1, MASP2*, and *VSIG4* variants were also independent of each other. In conclusion, polymorphisms compromising activation of the lectin pathway of complement increase susceptibility to HBV infection, with ficolin polymorphisms playing a major role in modulating the susceptibility among leprosy patients.

Keywords: mannose-binding protein-associated serine proteases, mannose-binding lectin, ficolin, leprosy, complement 3b receptors, genetic polymorphisms, complement system proteins, Hepatitis B

INTRODUCTION

Leprosy is an ancient disease that has plagued humanity through the ages. Although currently under control, it is still a big health problem in underdeveloped countries (1). Brazil's leprosy detection rate ranged from 10.9 to 78.4 per 100,000 inhabitants from 2001 to 2016, the second-highest rate worldwide. It is hyperendemic among children under 15 years of age, leaving a trail of irreversible disabilities behind, especially in boys (2).

Part of the susceptibility to leprosy and its broad spectrum of symptoms is genetically determined. Individuals with a strong cellular immune response can overcome the disease without even noticing the infection, whereas those who rely on a humoral response acquire a widespread disease that ultimately leads to blindness and gross deformities if left untreated (3). This is the cause of social stigmatization, which historically encouraged the isolation of affected individuals in "institutions" and "colonies". Although most of these institutions have been inactivated in Brazil, especially with the introduction of multidrug therapy (MTD), important remnants still present a high prevalence. They are historically recognized as former leprosy colonies, *e.g.*, the Prata Village, where MTD therapy resistance recently emerged (4).

Early exposure, late detection, and constant transmission of *Mycobacterium leprae* increase the probability of coinfections. In addition, confinement to a limited space is known to increase the spread of other infections (5–9). Furthermore, the same immune and genetic components that modulate the susceptibility to leprosy may modulate the susceptibility to coinfections, as well. This prompted our former epidemiological investigation on coinfections in South-Brazilian leprosy patients. Among those investigated, past infection with hepatitis B virus (HBV) emerged as the most frequent outcome (60%) compared to human immunodeficiency virus (HIV-1) (0.5%), hepatitis C virus (HCV) (3.5%), Chagas disease (4.5%), HTLV (0), and syphilis (9%) (5, 10). In fact, the frequency of HBV infection among leprosy patients was ten times higher than among the general Brazilian population (6%). In that study, it became clear that both diseases are strongly associated, and that institutionalization poses leprosy-affected individuals to a higher risk for HBV infection (10).

WHO estimates that 257 million individuals worldwide live with HBV (11). The worldwide prevalence of chronic HBV infection has been estimated at 3.9% (12), from about 0.7–1.6% in the Americas and Europe to 6.1–6.2% in African and Western Pacific regions, respectively. However, it reaches disproportionally high values in some Southeast Asia countries, as 10.8% in Vietnam (13). Among 50–80% of those chronically infected develop hepatocellular carcinoma, and 70–80% of these cases occur in patients with cirrhosis (14). Innate immunity factors that predispose to HBV infection and chronic complications are also involved in the susceptibility to leprosy and lepromatous disease, *e.g.*, polymorphisms of killer-cell immunoglobulin-like receptors (*KIR*) and human major histocompatibility complex class I chain-related gene A (*MICA*) genes (15–18).

Among innate immunological factors that drive susceptibility to infection and disease progression, the lectin pathway of complement (LP) occupies a prominent position. The LP starts typically with the recognition of sugar moieties or acetylated residues on pathogens or altered/damaged cells by the pattern recognition molecules (PRMs) such as the collectin mannan-binding lectin (MBL) or ficolins (FCNs), respectively. These pattern-recognition molecules are associated with homodimers of serine proteases (MASPs). When such PRM/MASP complexes bind to a surface, MASP-1 may autoactivate and then activates MASP-2. MASP-2 further cleaves complement factor C4, and MASP-1 and MASP-2 cleave C2 complement components, building up the complex of C4bC2a, which is a C3 convertase. This molecule cleaves C3 and channels the cascade further to cover C3b on the target, causing its opsonization, phagocyte internalization, and destruction. This step is a major outcome of complement activation, common to all three initiation pathways (including the classical and alternative pathways). The final steps of the proteolytic cascade lead to the assembly of the C5 convertase (C4bC2aC3b), subsequent cleavage of C5 and C5b aggregation of C6–C7 components, which pierce membrane-attack complexes through the addition of C8 and some of 10–18 C9 subunits on the target's surface [reviewed by (19, 20)].

The LP has long been recognized as playing a critical role in defense against various pathogens and has recently been

suggested as a target for controlling SARS-CoV-2 infections (21). Viral infections may spread opportunistically among already immunocompromised patients, regardless of their cause. Within this scenario, the LP's inflammation generating functions may act as a "double-edged sword"—low levels of its components may increase the chance for coinfections, whereas the opposite may accelerate tissue damage and aggravate the disease course. Leprosy poses a paradox to this problem since *M. leprae* relies on opsonization, one of the major outcomes of complement activation, to enter the host cell. Thus, it seems to usurp the LP to succeed, and deficiency/low levels of LP components have been associated with disease protection. The polarization of the disease in a relatively small proportion of individuals that do not get spontaneously cured places another layer of complexity to this immunopathological problem. The same genetic variants associated with resistance against leprosy may increase the risk to a lepromatous, Th2-associated clinical presentation or foster adverse treatment responses, as reversal (type 1) reactions and erythema nodosum leprosum (type 2) reactions (reviewed by 19).

Furthermore, individuals at higher risk for leprosy often share a low socio-economic background and hygiene environment that foster other infections. Past/present HBV infections in leprosy patients are not uncommon, reaching figures between 26 and 60% in Brazil (9, 10). However, as opposed to leprosy, a highly efficient immunological response associated with the complement cascade is necessary to abort infection. Notwithstanding, the same cascade may, as in the case of leprosy, cause more harm than good in chronic HBV infection and influence the clinical course towards adverse outcomes (22). This study enabled us to identify different, sometimes opposite associations comparing healthy, HBV-negative individuals and leprosy-affected, HBV-infected, and uninfected patients. This pioneering approach also brought new insights into the LP role and complement receptors on HBV infection and unprecedented findings on the genetic susceptibility to HBV/leprosy coinfection.

MATERIALS AND METHODS

Ethics Statement

All participants in this study were adults (over 18 years of age) and signed written informed consent, previously approved by the local medical ethics committee of the HC-UFPR (protocol 497.079/2002–06, 218.104 and 279.970). We designed this case–control, cross-sectional study and conducted it according to the Declaration of Helsinki.

Research Participants

We included 190 from a cohort of 199 leprosy patients who either attended the Federal University of Paraná's Clinical Hospital (HC-UFPR), Paraná's Hospital of Sanitary Dermatology (HDSPR) or the Regional Center of Specialities-Barão (CRS-Barão), as formerly described (10). We excluded nine individuals due to a lack of information for past/present HBV infection. All individuals were recruited in 2002–2003 when most of the Brazilian population was still not enrolled in the National Immunization Program for HBV vaccination. This program started in 1998 with a particular focus on newborn individuals (23). Of the 190 patients, 59 lived in the HDSPR at the recruitment time (31.1%).

According to the clinical classification proposed by Ridley and Jopling (24), 116 patients presented lepromatous leprosy (61.1%), 29 borderline leprosy (15.3%), 14 tuberculoid leprosy (7.4%), and 10 an undetermined form of leprosy (5.3%). Twenty-one (11.1%) were diagnosed with unspecified leprosy and excluded from any analysis comparing lepromatous and non-lepromatous patients.

After written informed consent, 7 ml of venous blood without anticoagulant was collected from each patient to test specific HBV antigens and antibodies in serum. Aliquots of 0.5–1 ml were used for each complement-quantifying assay. We also collected 4 ml of blood with anticoagulant ethylenediaminetetraacetic acid (EDTA) for DNA extraction from peripheral blood mononuclear cells through a commercial kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions.

The samples were tested for past/present HBV infection, as formerly described (10). Briefly, we identified anti-HBc (antibodies against HBV core antigen) using ELISA (MONOLISA ® a-HBc PLUS, BIO-RAD, Marnes La Coquette—France). This antibody is indicative of past HBV infection. We also searched for HBsAg, a marker of active HBV infection, with a sandwich ELISA (MONOLISA ® a-HBc PLUS, BIO-RAD, Marnes La Coquette—France). If positive for anti-HBc alone (HBsAg negative), patients were tested by microparticle enzyme immunoassay to detect antibodies against hepatitis B surface antigen (anti-HBs, a marker also used for positive vaccination response) (Murex anti-HBs, Murex Biotech Limited, Temple Hill, UK). All positive samples were retested using the same methods. Only five of 74 HBV positive individuals (6.8%) presented active HBV infection (positivity for HBsAg). Of these, two presented acute and three, chronic HBV infection. All others were positive for anti-HBc, indicative of a past resolved HBV infection. None was positive for anti-HBs alone, and none presented HBV-related liver cirrhosis or hepatocellular carcinoma. Of the 59 HDSPR-institutionalized patients, 36 were HBV+ (61%).

For practical reasons, we considered leprosy patients with past/present HBV infection as "HBV positive" (HBV+), whereas those negative for the tested markers, as "HBV negative" (HBV −). The HBV+ patients were older than HBV− patients, but the distribution of sex and ethnic groups did not differ among the patients (**Table 1**). We also included different groups of HBV− blood donors as control groups, depending on the investigated gene, from HC-UFPR, Hemepar, and Hospital Evangélico blood banks (**Table 2**). Their age and sex distributions were described before (25–31).

Identification and Characterization of Genetic Variants

We focused on LP genes plus phagocytic complement receptors for two principal reasons: (1) the LP gene products do not

TABLE 1 | Characterization of leprosy patients according to past/present HBV infection.

	Patients		P value	Lepromatous		P value	Non-lepromatous		P value
	HBV–	HBV+		HBV–	HBV+		HBV–	HBV+	
N	116	74		63	53		40	13	
Female (%)	47 (40.5)	25 (33.8)	ns	22 (34.9)	16 (30.2)	ns	22 (55.0)	6 (46.2)	ns
Average age (min–max)	50.5 (19–82)	57.1 (24–94)	0.0003	48.0 (20–73)	59.9 (30–94)	<0.0001	48.5 (19–75)	47.9 (31-67)	ns
Afro-Brazilian (%)	18 (15.5)	19 (25.7)	ns	9 (14.3)	14 (26.4)	ns	8 (20.0)	4 (30.8)	ns

Age distributions were compared with Student's t test, sex, and ethnic group distributions with Fisher's exact test. ns, not significative.

TABLE 2 | Sample sizes, genotyping method, and number of investigated polymorphism and haplotypes, per gene.

Genes	HBV– patients (ELISA/ TRIFMA)	HBV+ patients (ELISA/ TRIFMA)	Blood donors	Method	SNPs	Promoter	Coding (S/NS)	Intronic	UTR	eQTL	sQTL	Polymorph haplotypes (Total)	Reference
MBL2	116 (101, 30*)	74 (66, 16*)	200	Sanger Sequencing	19	14	4 (1/3)	0	1	3	0	8 (16)	(25)
FCN1	112	74	349	Multiplex SSP-PCR	7	6	0	0	1	5	1	7 (10)	(26)
FCN2	83	56	131	Sanger Sequencing	9	5	3 (1/2)	0	1	5	7	6 (8) 5 (5)	(27)
FCN3	92 (37)	51 (31)	146	Multiplex SSP-PCR	3	0	1 (frameshift)	2	0	3	2	3 (5)	(28)
MASP1	108 (79)	72 (60)	196	Multiplex SSP-PCR	5	0	0	2	3	4	1	10 (12)	(29)
MASP2	112 (22)	73 (24)	408	Multiplex SSP-PCR	11	1	6 (0/6)	3	1	9	8	9 (17)	(30)
CR1	96 (12)	56 (8)	198	Multiplex SSP-PCR	9	0	3 (0/3)	4	2	8	5	13 (15)	(31)
VSIG4	111	74	177	Multiplex SSP-PCR	6	2	1 (0/1)	3	0	4	0	4 (7)	—
C3	93 (49)[&]	60 (28)[&]	191	Multiplex SSP-PCR	3	0	2 (0/2)	1	0	3	0	4 (5)	—

SNP, single nucleotide polymorphism (S, synonymous; NS, non-synonymous); eQTL and sQTL, expression and splicing quantitative trait loci for the same or other gene, respectively (data from GTEx portal).

**MBL/C4 complexes (measure of LP activation).*

[&]Evaluated with an immunoturbidimetric assay.

MBL2, mannan-binding lectin 2; FCN, ficolin; MASP, MBL-associated serine protease; CR1, complement receptor 1; VSIG4, V-set and immunoglobulin domain containing 4; C3, complement component 3.

SSP-PCR, sequence-specific polymerase chain reaction.

primarily rely on antibodies and thus on the adaptive immune response to launch the complement cascade, as is the case for the classical pathway. It is thus the first to get activated after pathogen recognition, also playing an important role in activating the alternative pathway (through MASP-3); (2) PRMs of the LP readily recognize *Mycobacterium leprae* as well as HBV, mediating their phagocytosis through the investigated phagocytic receptors (which in the case of *M. leprae* may be beneficial for the parasite and establishment of infection). Furthermore, we chose SNPs whose alleles were: common in at least one of the major human ethnic groups and were either: (1) located in a regulatory region and associated with gene expression/protein levels, or (2) located in an exon and causing a structural/functional defect in the protein due to a missense or indel mutation.

We identified 72 single nucleotide polymorphisms (SNPs) of nine genes of the complement system (**Table 2**). Four genes that encode PRMs (*MBL2*—mannan-binding lectin, *FCN1*—M-ficolin, *FCN2*—L-ficolin and *FCN3*—H-ficolin), two that encode collectin and ficolin-associated serine proteases (*MASP1* and *MASP2* – MBL-associated serine proteases), two that encode complement receptors (*CR1*—complement receptor 1 or CD35, *VSIG4*—V-set and immunoglobulin domain-containing 4 or CRIg), and one encodes the complement component C3 (*C3*). Among the selected 72 polymorphisms, 39% occur in the promoter region, 28% in exons (25% are non-synonymous substitutions), 21% in introns, and 13% in 5′ or 3′ untranslated regions (UTRs). Of all of these, 61.1% (44/72) are associated with mRNA levels and 33.3% (24/72) with alternative splicing of the pre-mRNA of the same gene or a neighboring gene (expression quantitative trait loci or eQTL and splicing quantitative trait loci or sQTL, respectively), according to data of the GTEx portal (https://gtexportal.org). These variants were distributed in 100 haplotypes, of which 69 are polymorph (with more than 1% frequency in blood donors) (**Table 2**).

We used Sanger sequencing to identify *MBL2* and *FCN2* genetic variants and multiplex sequence-specific PCR (SSP-PCR) to identify *FCN1, FCN3, MASP1, MASP2*, and *CR1* polymorphisms, as formerly described (25–31). Multiplex-SSP-PCR was also the choice technique for identifying C3 and *VSIG4*

polymorphisms (primer sequences and PCR protocols are available in **Supplementary Table 1**).

Measurement of Complement Proteins

MBL serum concentrations were measured in a double-antibody enzyme-linked immunosorbent assay (ELISA), with an in-house assay previously described by our group (32). Serum FCN-3 and MASP-2 concentrations and serum MBL-C4 activation complexes were measured as described before (19, 28, 30) with commercial ELISA kits (HK340, HK326, and HK327, respectively, from Hycult Biotechnology, Uden, The Netherlands). Serum sCR1 levels were measured as described before (31) by a commercial enzyme-linked immunosorbent assay (ELISA), using the SEB123Hu kit (USCN Life Science Inc., Wuhan, China), according to the manufacturer's instructions. Color intensity was evaluated at 450 nm in an ELISA reader.

Serum MASP-1, MASP-3, and MAp44 levels were measured with time-resolved immunofluorimetric assays (TRIFMA), as published (29). In the MASP-3 and MAp44 TRIFMA, the bound protein is detected by a specific biotin-labeled monoclonal antibody, recognized by europium-labeled streptavidin. The provided signal is measured by time-resolved fluorometry (33). For measuring MASP-1, we used an inhibition assay. Circulating MASP-1 from the sample inhibits the binding of an anti-MASP-1 antibody to a surface coated with a fragment of MASP-1, followed by its detection, as previously described (34).

C3 serum levels were assessed using the automated immunoturbidimetric assay (Beckman Coulter, AU analyzer, USA) with reference values ranging from 87 to 200 mg/dl, the according to manufacturer's protocol (Beckman Coulte, ref: OSR6159).

Statistics

Genotype, allele, and haplotype frequencies were obtained by direct counting and compared using Fisher's exact test and odds ratios with the respective 95% confidence limits. The expectation-maximization (EM) algorithm was used to reconstruct haplotypes not phased by SSP primers and calculate maximum likelihood estimates of haplotype frequencies while considering phase ambiguity. The EM calculations and the hypothesis of Hardy–Weinberg equilibrium test were performed with the ARLEQUIN software package version 3.1 (http://cmpg.unibe.ch/software/arlequin3/). We compared the distribution of protein serum concentrations between the groups, using non-parametric Mann–Whitney/Kruskal–Wallis tests (since their distribution did not pass the Shapiro–Wilk normality test), with Graphpad Prism 5.01 (GraphPad Software, La Jolla, CA).

The reduced multivariate logistic regression model was used to adjust results for demographic factors using STATA v.9.2 (StataCorp, TX, USA). The P values obtained with multiple association tests were corrected with the Benjamini–Hochberg method (35). We ended up with *cc.* 75% statistical power for most comparisons between common haplotypes with at least 20% frequency in HBV− leprosy patients and 10% in HBV+ patients (for uncommon haplotypes with 1 and 5% in each of these groups, respectively, it was 65%).

We used the following strategy for data interpretation: if the results comparing healthy controls with HBV+ individuals did not differ from the comparison between HBV+ and HBV− leprosy patients, we considered the association as independent of *M. leprae* infection and due to a higher susceptibility or resistance to HBV *per se*. This first analysis allowed us to disentangle HBV infection susceptibility from susceptibility to HBV/*M. leprae* coinfection. Results specific to the comparison within the leprosy group, between HBV+ and HBV− individuals, corresponded to increased susceptibility or resistance to both diseases. Finally, we also focused on possible associations within the lepromatous and non-lepromatous groups, which is particularly interesting due to their contrasting Th1 and Th2 immune responses, respectively.

Associated polymorphisms were further characterized according to data of the ENCODE (Encyclopedia of Noncoding Elements), available in the UCSC Genome Browser (https://genome.ucsc.edu/), Polyphen and SIFT scores from the Ensembl Genome Browser (https://www.ensembl.org/).

RESULTS

By comparing HBV- and leprosy- blood donors, we were able to identify gene associations due to susceptibility to HBV *per se* and differentiate them from genetic associations with susceptibility to leprosy only [already published in references (25–31)], and to both HBV and leprosy. The last may be interpreted as increased susceptibility to HBV infection in the already immunocompromised leprosy-patient group (coinfection) or higher HBV susceptibility of leprosy-prone individuals (HBV infection before *M. leprae* infection).

Associations With Hepatitis B Virus Infection *Per Se*

We considered the following genetic associations as with HBV infection *per se* (independent from leprosy disease) if the direction of association did not differ between the comparisons of HBV+ leprosy patients and blood donors and the comparisons between HBV+ and HBV− leprosy patients, either at the level of haplotype frequencies (**Supplementary Tables 2–11**) and/or at the level of genotype frequencies (**Table 2**). Seven haplotypes of six different genes were associated with a dominant or an additive effect on the susceptibility or resistance to HBV infection after correcting for age and ethnic group distribution. We found no associations with protein serum levels of any tested complement component (**Supplementary Figure 1**) or tested *C3* polymorphisms (**Supplementary Table 11**).

Five frequent haplotypes were associated with increased susceptibility to HBV infection. Among them, as well as among all selected genes, the polymorphism of *MBL2* is particularly outstanding due to its common deficiency-causing variants. One of them is embedded in the *LYQC* haplotypes, associated with the disease in the multivariate logistic regression, independently of age and ethnic group distribution (OR = 3.38

[95%CI = 1.19–9.57], P = 0.022) (**Table 3**). Indeed, the summed frequencies of three different *LYQC* haplotypes identified by haplotype-specific sequencing did not differ between controls, HBV– leprosy, and HBV– lepromatous patients (**4F1-l, *4F2A-l* with rs45602536 and **4F3-l* with rs67990116 minor promoter alleles) (**Supplementary Table 2**). All *LYQC* haplotypes carry the minor allele of the rs1800451 polymorphism in codon 57, which disrupts the collagen Gly-Xaa-Yaa repeats by causing the substitution of glycine with glutamic acid (p.Gly57Glu, called the "*C* allele"). They also present five "*Q*" variants in almost absolute linkage disequilibrium in all human populations, occurring within a topologically associated chromatin domain (TAD): *rs7095891*T, rs11003124*C, rs7084554*G, rs36014597*G, rs10556764*DelAAAGAG*, and *rs11003123*T*. The *rs7095891*T* (classical "*Q*" allele) also disrupts a CpG site. Eighteen regulatory proteins bind to the DNA sequence containing this variant and the *rs11003123*T* allele. Among them, the forkhead box proteins FOXA1 and FOXA2

TABLE 3 | Association of demographic and genetic variables with past/present HBV infection in leprosy patients.

Variables	Association	Univariate	Analysis		Multivariate	Analysis &		Reduced	Model		
LE HBV– *vs* HBV+	**Model**	**OR**	**(95%CI)**	**p**	**OR**	**(95%CI)**	**p**	**OR**	**(95%CI)**	**p**	**P corr.**
Age		1.04	(1.01–1.06)	<0.0001				1.06	(1.02–1.1)	0.002	0.008
Ethnicity		0.53	(0.26–1.1)	0.087							
MBL2*LYQC	Additive	3.49	(1.29–9.44)	0.014	**3.38**	**(1.19–9.57)**	**0.022**				
FCN1*1	Additive	1.68	(1.08–2.60)	0.021	**1.66**	**(1.05–2.63)**	**0.029**				
FCN1*3C2	Additive	0.57	(0.34–0.95)	0.03	**0.56**	**(0.33–0.95)**	**0.033**				
FCN2*GGGCAC	Dominant	3.53	(1.32–9.43)	0.012	**2.96**	**(1.06–8.25)**	**0.038**	**6.73**	**(1.66–27.35)**	**0.008**	**0.025**
*FCN2*MAG*	Dominant	0.51	(0.24–1.09)	0.084	0.62	(0.28–1.38)	0.242				
*FCN2*TA*	Additive	1.34	(0.89–2.02)	0.162	1.22	(0.79–1.88)	0.371				
FCN3*Del Del C	Dominant	7.74	(0.84–71.27)	0.071	**9.59**	**(1.02–90.34)**	**0.048**	**12.54**	**(1.16–135.75)**	**0.037**	**0.050**
MASP1*AC_CC	Dominant	2.88	(1.40–5.92)	0.004	**2.61**	**(1.22–5.58)**	**0.013**	**3.99**	**(1.31–12.21)**	**0.015**	**0.042**
*MASP1*AC_CTG*	Dominant	0.15	(0.02–1.25)	0.08	0.14	(0.02–1.20)	0.074				
MASP2*1C2-I	Dominant	3.85	(0.96–15.42)	0.057	**5.35**	**(1.22–23.27)**	**0.026**				
MASP2*2B1-i	Dominant	4.29	(1.29–14.24)	0.017	**4.48**	**(1.29–15.53)**	**0.018**	**19.25**	**(2.73–135.93)**	**0.003**	**0.017**
CR1*3A	Dominant	2.52	(1.24–5.14)	0.011	**2.65**	**(1.25–5.62)**	**0.011**				
VSIG4*TGGRCG	$	6.60	(1.36–32.05)	0.019	**10.00**	**(1.94–51.64)**	**0.006**	**12.55**	**(1.65–95.24)**	**0.014**	**0.033**
LL HBV– *vs* HBV+	**Model**	**OR**	**(95%CI)**	**p**	**OR**	**(95%CI)**	**p**	**OR**	**(95%CI)**	**p**	**P corr.**
Age		1.05	(1.02–1.08)	<0.0001				1.06	(1.02-1.11)	0.004	0.01
Ethnicity		0.46	(0.18–1.18)	0.107							
*MBL2*HYPA*	Additive	0.65	(0.36–1.16)	0.145	0.76	(0.40–1.43)	0.392				
MBL2*LYQC	Additive	3.94	(1.06–14.67)	0.041	3.47	(0.84–14.41)	0.087	**8.82**	**(1.02–76.59)**	**0.048**	**0.05**
FCN1*1	Additive	1.78	(1.02–3.09)	0.041	1.69	(0.93–3.07)	0.083	**2.71**	**(1.07–6.88)**	**0.036**	**0.03**
*FCN1*3C2*	Additive	0.53	(0.28–1.04)	0.064	0.61	(0.29–1.27)	0.189				
*FCN2*AGAAGC*	Additive	0.40	(0.18–0.89)	0.024	0.44	(0.18–1.08)	0.072				
*FCN2*GGGCAC*	Dominant	2.5	(0.78–8.05)	0.125	1.49	(0.42–5.31)	0.538				
FCN2*MAG	Dominant	0.23	(0.08–0.63)	0.005	**0.29**	**(0.10–0.86)**	**0.026**				
FCN2*TA	Additive	2.30	(1.30–4.06)	0.004	**2.15**	**(1.16–4.0)**	**0.015**	**2.57**	**(1.27–5.22)**	**0.009**	**0.02**
MASP1*AC_CC	Dominant	4.45	(1.60–12.38)	0.004	**4.99**	**(1.63–15.21)**	**0.005**				
MASP2*1C2-I	Dominant	6.35	(0.72–56.21)	0.096	**10.09**	**(1.01–100.6)**	**0.049**				
MASP2*2B1-i	Dominant	3.13	(0.58–16.82)	0.185	4.47	(0.69–29.18)	0.118	**19.71**	**(1.03–378.7)**	**0.048**	**0.04**
*CR1*3A*	Dominant	2.00	(0.82–4.88)	0.129	2.05	(0.78–5.43)	0.148				
*VSIG4*TGACTA*	Dominant	0.50	(0.20–1.23)	0.129	0.58	(0.21–1.65)	0.311				
NL HBV– *vs* HBV+	**Model**	**OR**	**(95%CI)**	**p**	**OR**	**(95%CI)**	**p**	**OR**	**(95%CI)**	**p**	**P corr.**
*MBL2*HYPA*	Recessive	7.09	(0.59–85.7)	0.123							
*MBL2*LYQC*	Dominant	3.7	(0.65–21.21)	0.142							
*FCN2*GGGCAC*	Dominant	6.75	(0.92–49.67)	0.061							
MASP1*AC_CC	Dominant	6.0	(1.37–26.24)	0.017				**7.15**	**(1.1–46.46)**	**0.039**	**0.05**
*MASP2*2B1-i*	Dominant	6.0	(0.87–41.44)	0.069							
CR1*3A	Additive	5.32	(1.48–19.12)	0.010				**5.06**	**(1.28–20.05)**	**0.021**	**0.025**

LE, Leprosy patients; LL, Lepromatous leprosy; NL, Non-lepromatous leprosy; HBV+, with past or present hepatitis B infection, as judged by positive HBsAg and/or anti-HBc and anti-HBs antigen serological results; OR, odds ratio; CI, confidence interval; p, two-tailed p value.

MBL2, mannan-binding lectin; FCN, ficolin; MASP, MBL-associated serine proteases; CR1, complement receptor; VSIG4, V-set and immunoglobulin domain containing 4.

Genetic variants to be tested in the univariate analysis were selected comparing haplotype distributions between HBV+ and HBV– individuals in any of the leprosy, lepromatous and/or non-lepromatous groups, according to a significant result of the exact Fisher test (two-tailed P value < 0.5). Only selected variables are shown.

P values equal or lower than 0.2 in the multivariate logistic regression, after correction for age and ethnicity, were used as threshold for inclusion of variables in the final model of multivariate regression.

& Corrected for age and ethnicity.

In bold: associations with genetic variants that remained significant, after correction for age and ethnicity and/or in the final reduced logistic regression model and after correction for multiple comparisons with the Benjamini–Hochberg method (P corr).

Underlined: amino acid one-letter symbols (shown in the case of missense mutations).

% considering all haplotypes associated with low MASP2 expression:

$ VSIG4 is X-linked. In this model, we considered both male and female genotypes, but did not consider as "TGGRCG" positive, female patients with heterozygous TGGRCG/TGARTA genotypes (TGARTA seems to be associated with a protective effect against leprosy per se—Stinghen et al., manuscript in preparation).

(hepatocyte nuclear factors 3 alpha and beta, respectively) and the histone acetyltransferase EP300 have the strongest affinity (ChIpSeq chromatin immunoprecipitation data from the ENCODE project, available in the UCSC Genome Browser for the HepG2 liver cell line). Interestingly, we found a trend for an association of the *LYPA* haplotype with leprosy *per se* (OR = 2.04 [95%CI = 0.98––4.28], P = 0.062), as formerly published (**Supplementary Table 2**) (25).

Among the seven investigated *FCN1* SNPs, all of which occur within a TAD, the combination of the most 5′ five alleles (*AAAGDelT*), present in the **3B2, *3C1, *3C2*, and **3C2.3A* haplotypes, occurred with higher frequency among blood donors than among HBV+ leprosy patients (OR = 0.6 [95%CI = 0.39–0.9], P = 0.014). The same protective association sustained with the addition of the next 5′ variant to the analysis (*rs10117466*A*), including only the summed frequencies of the **3C2* and **3C2.3A* haplotypes, for the comparison between HBV− and HBV+ leprosy patients (OR = 0.58 [95%CI=0.36–0.94], P = 0.033) (**Supplementary Table 3**). Among these haplotypes, the common *FCN1*3C2* haplotype presents five allelic variants associated with higher *FCN1* gene expression (*rs2989727*A, rs10120023*A, rs28909976*delT, rs10117466*A* and *rs10858293*T*) and three (*rs2989727*A, rs10120023*A, rs17039495*A*) that also disrupt a potentially methylated CpG site. Not surprisingly, this haplotype remained associated with resistance against HBV infection, independently of age and ethnic group distribution (OR = 0.56 [95%CI = 0.33–0.95], P = 0.033) (**Table 3**). In contrast, the frequency of the **3A.3C2.B* haplotype, which presents three variants associated with lower FCN-1 expression (*rs10120023*G, rs28909976*insT* and *rs10117466*C*), was higher among HBV+ leprosy patients than among blood donors (OR = 3.64 [1.25–10.67], P = 0.023) (**Supplementary Table 3**).

There were no *FCN2* or *FCN3* haplotypes associated with HBV infection (**Supplementary Tables 4–6**) although we confirmed the protective association of the *FCN2* promoter-exon 1 haplotype *AGAAAC* with leprosy *per se* (OR = 0.13 [95% CI=0.02–0.99], p = 0.018) (**Supplementary Table 4**) (27).

Polymorphisms of both MBL-associated serine protease-encoding genes were associated with HBV infection. We also confirmed the recently published association with leprosy disease and the *MASP1*GC_CCG* haplotype, obtained with the same group of patients (OR = 1.81 [95%CI = 1.25–2.64], P = 0.002) (29). Furthermore, we identified an opposite association with the *MASP1*GC_CCA* haplotype, with leprosy resistance (OR = 0.50 [95%CI = 0.28–0.89], P = 0.019). The summed frequencies of *MASP1*AC_CC* haplotypes, diverging only at the most 3′ *rs850314*G>A* polymorphism, were higher among HBV+ leprosy patients compared to blood donors or HBV− leprosy patients (**Supplementary Table 6**). Susceptibility was associated with these haplotypes, independently of age and ethnicity, as well as of all other genetic associations (OR = 3.99 [95%CI = 1.31–12.21], P = 0.015) (**Table 3**).

For the *MASP2* gene, we confirmed the previously published association of the *MASP2*2B1-i* haplotype (regardless of the intron 4 rs2273344–intron 5 rs9430347 *AG* or *GA* combinations) with resistance against leprosy *per se* (OR = 0.29 [95%CI = 0.10–0.82], P = 0.009) (**Supplementary Table 8**) (30). In contrast, the same haplotypes were associated with susceptibility to HBV infection, independently of other genetic associations, age, and ethnic group distribution (OR = 19.25 [95%CI=2.73–135.93], P = 0.003) (**Table 3**). Furthermore, the **2B2B-l* haplotype, carrying the deficiency caused by the p.Asp120Gly variant (rs72550870), was first revealed as associated with leprosy disease. This result corroborates previous observations that low MASP-2 levels may raise the risk of *M. leprae* successful infection (31) (**Supplementary Table 8**). Another MASP-2 deficiency-causing variant was also associated, but with susceptibility to HBV *per se*, independently of age and ethnicity. It substitutes arginine by histidine at the 439th amino acid position within the activation site of the serine protease domain, being encoded by the **1C2-l* haplotype (OR = 5.35 [95%CI = 1.22–23.27], P = 0.026) (**Table 3**).

Polymorphisms of both investigated complement receptor genes were associated with the HBV infection. Regarding *CR1*, there was a trend for a higher frequency of the **3A1* haplotype among blood donors than among HBV+ leprosy patients (OR = 2.33 [95%CI = 0.88–6.16], P = 0.087) (**Supplementary Table 9**). In fact, the summed frequencies of all **3A* haplotypes were associated with increased susceptibility to HBV infection, independent of age and ethnic group distribution (OR = 2.65 (95%CI = 1.25–5.62), P = 0.011) (**Table 3**). They differ from all other *CR1* haplotypes by presenting arginine instead of histidine at the 1,208th amino acid position of the CR1 protein, a missense mutation deemed as probably damaging for at least one of the *CR1* transcripts (Polyphen score of 0.98).

Although none of the X-linked *VSIG4* eQTL variants seem associated with altered gene expression/structure of *VSIG4* itself (only of neighboring genes), major haplotypes were found associated with HBV infection *per se*. The summed frequencies of two haplotypes carrying *rs5964488*G (p.1208Arg), rs34581041*C, rs5964487*C*, and *rs9887348*G*, all of which occur within TADs, were higher among HBV+ leprosy patients than among blood donors (OR = 2.11 [95%CI = 1.23–3.61], P = 0.009) and HBV− leprosy patients (OR = 1.93 [95%CI = 1.08–3.45], P = 0.034) (**Supplementary Table 10**). One of them (*VSIG4*TGGRCG*) remained associated with HBV infection, independent of any other associated factor (OR = 12.55 [95% CI = 1.65–95.24], P = 0.014) (**Table 3**). Conversely, frequencies of the most common *TGARTA* haplotype were lower among HBV+ leprosy patients than among blood donors (OR = 0.56 [95%CI = 0.33–0.94], P = 0.037) and HBV− leprosy patients (OR = 0.54 [95%CI = 0.30–0.91], P = 0.038) (**Supplementary Table 10**).

Associations With Hepatitis B Virus Infection in Leprosy Patients

Three ficolin haplotypes were associated with HBV infection, restricted to the group of leprosy patients only. Although the *MASP1*AC_CTG* presented a trend to a leprosy-restricted HBV association as well (**Supplementary Table 7**), this was not confirmed after correction for age and ethnic group distributions (OR = 0.14 [95%CI = 0.02–1.20], P = 0.074) (**Table 3**).

Compared to *FCN1*3C2* haplotypes, associated with higher *FCN1* expression and an additive effect on resistance against HBV infection *per se*, the most ancestral *FCN*1* haplotype carries three major allelic variants forming CpG sites (*rs2989727*G, rs10120023*G*, and *rs17039495*G*) and at least four variants associated with lower *FCN1* gene expression (*rs10120023*G*, *rs28909976*insT, rs10117466*C*, and *rs10858293*G*). Not surprisingly, this haplotype was associated with higher susceptibility to leprosy/HBV infection, independently of age and ethnicity (OR = 1.66 [95%CI = 1.05–2.63], P = 0.029) (**Table 3**, **Supplementary Table 3**).

Regarding *FCN2* promoter-exon 1 haplotypes, those two sharing the first three 5′ *rs3124952*G, rs3124953*G*, and *rs3811140*G* (*GGG*) variants were also associated with increased susceptibility to leprosy/HBV infection (OR = 2.6 [95%CI = 1.13–5.96], P = 0.034) (**Supplementary Table 4**). In particular, *GGGCAC* remained associated with susceptibility to both diseases, independently of any other associated factor (OR = 6.73 [95%CI = 1.66–27.35], P = 0.008) (**Table 3**). Interestingly, the first two 5′ *rs3124952*G* and *rs3124953*G* variants of these haplotypes enhance splicing of the second exon (increasing the amount of the ENST00000350339.3 transcript, which encodes a smaller protein of 275 amino acids, presenting a shorter collagen domain). Furthermore, the last but one *rs17514136*A* variant within these two haplotypes is associated with lower *FCN2* expression (data from the GTEx portal). Since linkage disequilibrium is negligible between promoter-exon 1 and exon 8 *FCN2* variants, we separately evaluated the exon 8 variants encoding the fibrinogen domain. The frequencies of *TA* haplotypes (composed by rs17549193 and rs7851696 encoding the ancestral amino acids threonine at position 236 and alanine at position 258 of the ficolin-2 protein, respectively) were higher among HBV+ than among HBV− leprosy patients (OR = 2.19 [95%CI = 1.14–4.23], P = 0.022). On the other hand, *MAG* haplotypes (with methionine at position 236) were associated with protection against both diseases (OR = 0.36 [95%CI = 0.17–0.77], P = 0.011) (**Supplementary Table 5**). Although methionine and threonine seem to be equally well tolerated at amino acid position 236, the *rs17549193*T* variant (creating the Met codon) also disrupts a CpG site and is associated with higher *FCN2* expression. Nevertheless, exon 8 variant associations did not resist correction by age and ethnic group distribution within our setting (**Table 3**).

Lastly, the *FCN3*del_del_C* haplotype carrying two deletions, one in exon 5, causing a frameshift in the protein and FCN-3 deficiency (rs532781899) and another one in intron 5 (rs28362807), was associated with a dominant effect for increased susceptibility to both diseases, independent of any other associated factor (OR = 12.54 [95%CI = 1.16–135.75], P = 0.037) (**Table 3**, **Supplementary Table 6**).

Associations With Hepatitis B Virus Infection in Lepromatous and Non-Lepromatous Patients

Despite the lack of statistical power in the two lepromatous and non-lepromatous subgroups, several of the susceptibility associations mentioned above were also found with HBV/lepromatous leprosy (*MBL2*LYQC, FCN1*1, MASP1*AC_CC, MASP2*1C2-l*, and *MASP2*2B1-i*) and with HBV/non-lepromatous leprosy (*MASP1*AC_CC* and *CR1*3A*) (**Supplementary Tables 2**, **3**, **7**–**9**, **Table 3**). The most severely affected lepromatous group also featured an opposite association with the *FCN2*MAG* (OR = 0.29 [95%CI = 0.10–0.86], P = 0.026) and *FCN2*TA* (OR = 2.57 [95%CI = 1.27–5.22], P = 0.009) haplotypes, independent of age and ethnic group distributions (**Table 3**). Nevertheless, some associations were lost after multivariate logistic correction (*FCN1*3C2, FCN2*AGAAGC*, and *FCN2*GGGCAC* with lepromatous leprosy/HBV infection and *MBL2*HYPA*, *MBL2*LYQC, FCN2*GGGCAC* with non-lepromatous leprosy/HBV infection) or due to low sample size (*MASP1*AC_CTG*, *FCN3*2B2.2A*) (**Supplementary Tables 2**–**4**, **6**, **Table 3**).

DISCUSSION

Evidence from growing research on HBV infection complications as liver cirrhosis, hepatocellular carcinoma, and acute-to-chronic liver failure shows a critical role for complement activation in the disease. In chronic hepatitis B patients, HBV sensitizes hepatocytes to complement-mediated killing through down-regulation of the complement-regulating membrane protein CD59, causing liver inflammation, and clearing the virus (36). Conversely, viral persistence seems associated with HBV-driven suppression of both gene expression and protein levels of complement components 2, 3, and 4 (37–40), added to lower complement activation (39). Lower gene/protein expression of these components may be understood as a simple byproduct of liver cell death and loss of healthy hepatic tissue, which is the primary source for most soluble complement proteins. Instead, we found evidence that common innate deficiency of complement proteins, manifesting either as lower serum concentrations or lower functional efficiency, predisposes to HBV infection, sometimes dependent on leprosy disease. This is the first time that the genetic susceptibility to HBV infection is investigated among leprosy patients, which are already at risk due to stigmatization and institutionalization-driven aggregation (10). From the comparison with HBV- blood donors (which unfortunately were not the same for all investigated genes, not allowing for multivariate analysis), we disentangled general associations from those particular of leprosy or even lepromatous, patients. Our approach revealed a previously unsuspected stratification within our leprosy patient group, caused by past/present HBV infection, clearly associated with institutional confinement (10). This hidden stratification may affect other patient groups as well, especially those confined to an environment that eases the spread of highly infectious diseases, as HBV.

There is a general lack of understanding regarding the role of the LP and its components and genetic variants in HBV infection and chronic disease. Most investigations refer to common *MBL2* polymorphisms, in sometimes conflicting reports starting at the late nineties (41–45). From a metanalysis including 17 studies, of

which two were Brazilian (46, 47), the authors concluded that exon 1 variants leading to MBL deficiency (p.Gly54Asp, rs1800450; p.Gly57Glu, rs1800451, and p.Arg52Cys, rs5030737; known as *B, C*, and *D* variants and collectively named as *O* variants) have a dominant effect on the susceptibility to severe hepatitis B or liver cirrhosis, but not to chronic hepatitis B and hepatocellular carcinoma (48). In another Brazilian study, patients with active HBV infection and homozygous for the fully functional exon 1 MBL *A* variants presented a positive correlation between increased transaminase and HBV DNA levels and the presence of mild to moderate fibrosis (49).

In our study, we identified an association of *C*, but not of *D*, and *B*-carrying haplotypes with higher susceptibility to HBV infection. Interestingly, the *C* variant was also more frequent in non-responders to HBV vaccination in African adults (50). The reasons for an association restricted to the *LYQC* haplotypes and independent of ethnic distribution (the *C* variant is much more frequent among the African and Afro-Brazilian populations (51, 52) may be several. The deficiency associated with *O* variants is commonly taken for granted due to a profound effect on the protein's assembly and stability, which leads to an increase of low-molecular-mass MBL that has reduced the capacity of activating complement and of ligand binding (53, 54). Nevertheless, the protein with the analogous *C* variant in rats is 10 times less efficient in activating the LP than the analogous *B* variant (55). Furthermore, the *C* variant occurs in absolute linkage disequilibrium with six so-called "*Q*" variants, which share a positive selection signature in the promoter–3′UTR region (52). Screening ENCODE ChIp-Seq data, we found 18 regulatory proteins binding to this TAD region. Those three with the highest affinity are strong hepatocyte-activating factors. This explains former findings that unequivocally associated *Q* variants with higher *MBL2* gene expression (51, 56–58). Thus, the associated *LYQC* haplotypes produce a protein with a profound defect on LP activation and probably also produce it in much higher amounts than the other *O*-harboring *LYPB* and *HYPD* (and the less common *LYPD* haplotypes) (59).

With the present study, we were also the first to demonstrate an association between *FCN1* haplotypes and HBV infection susceptibility. This gene encodes the only PRM of the LP that can be either membrane-bound or soluble, localized in gelatinase granules in the cytoplasm of neutrophils, secretory granules in monocytes, and type II alveolar epithelial cells in the lung (reviewed by 56). Haplotypes with the *AAAGDelT* combination are associated with higher *FCN1* gene expression and were associated with protection against HBV infection, particularly *FCN1*3C2.* On the other hand, the *FCN1*3A.3C2.B* haplotype and the more ancient *FCN*1* haplotype were associated with lower *FCN1* gene expression and higher susceptibility to HBV and leprosy/HBV infection, respectively. This opposite association agrees with the hypothesis that the LP's efficient activation may block HBV spread in the host.

FCN2 polymorphisms did not emerge as associated with HBV infection itself, but only in the context of leprosy disease, indicating that this PRM probably plays a role in modulating the susceptibility to HBV within the context of the already compromised immune response of *M. leprae* infected individuals. Considering the long incubation time between *M. leprae* infection and disease manifestation decades later, this situation may be expected. In accordance, six of the same *FCN2* polymorphisms investigated in this study were also not associated with the HBV vaccinal humoral immune response (50). The susceptibility association with the promoter-exon 1 *rs3124952*G, rs3124953*G*, and *rs3811140*G* (*GGG*) combination and foremost of the *GGGCAC* haplotype is best explained by the associated differential splicing of the second exon and consequent production of a protein with a shorter collagenous domain, as well as with lower *FCN2* expression (data from the GTEx portal). Variant combinations of exon 8 (encoding the fibrinogen domain) were also associated with both diseases, but only within the lepromatous group. In this case, the ancestral amino acid sequence increased the susceptibility, whereas methionine at position 236 was associated with higher *FCN2* expression and protection. Interestingly, serum ficolin-2 concentrations were higher in chronic HBV patients than in healthy controls and HBV carriers and decreased with positive treatment response, but was lowest in individuals with hepatocellular carcinoma and cirrhosis (60).

In agreement with the previously mentioned associations with polymorphisms of PRM genes from the LP, another well-known deficiency-causing haplotype harboring the rs532781899 frameshift and causing a truncated ficolin-3 protein was associated with HBV/leprosy. This is the first time that any *FCN3* variant is associated with HBV disease. As with *FCN2* polymorphisms, the association was restricted to leprosy patients with a dominant effect, meaning that heterozygote individuals are already at higher risk. To our knowledge, the only investigation previously done revealed differential FCN-3 protein expression in HBV-related hepatocellular carcinoma (61).

Compared to the PRM-encoding genes, those encoding the LP serine proteases are highly pleiotropic, challenging the interpretation of association results. For example, *MASP1* produces at least three proteins and several predicted transcripts (Ensembl, 2020). Among the proteins, MASP-1 and MASP-3 have different catalytic domains and non-catalytic MAp44 acts as a complement regulatory protein in myocardial tissue. MASP-1 autoactivates, activates MASP-2, and cleaves C4, playing a critical role in LP activation. It is a promiscuous enzyme, also cleaving other proteins, as prothrombin in the coagulation cascade. On the other hand, MASP-3 competes with MASP-1 for the same recognition sites in the collagenous PRM stalks but cannot activate the LP. Instead, it cleaves pro-factor D from the PRM-independent alternative pathway (AP) in "resting" blood (under non-inflammatory conditions) [reviewed by (62)]. The *MASP1*AC_CC* haplotype associated with higher susceptibility to HBV infection presents a 3′UTR combination in exon 12 that possibly acts as a target for miRNAs, being also associated with higher MASP-3 and lower MASP-1 levels (29). Thus, the association of this *MASP1* haplotype with HBV susceptibility may be explained by blockage/hindered LP activation, as well as

excessive AP activation. Interestingly, in HBV-chronically infected patients that undergo acute-on-chronic liver failure, MASP-1 production was found repressed, whereas MASP-2 was up-regulated—being both mostly produced in the liver (63).

The *MASP2* gene produces the MASP-2 serine protease and high amounts of the truncated MAp19 protein in the liver, whose function is still unclear [reviewed by (62)]. Regarding MASP-2, our results are in line with our previously published results in HIV+HBV+ individuals, whose serum levels were lower than in controls. The p.126L variant associated with MASP-2 levels <200 ng/ml increased the susceptibility to HIV+ HBV+ status (19). Interestingly, the MASP2*p.439H variant, imbedded in the **1C2-l* haplotype and associated with increased susceptibility to HBV infection in this study, occurs in absolute linkage disequilibrium with p.126L. This haplotype was also formerly found associated with leprosy *per se* (30). The resulting MASP-2 protein can bind to PRMs but cannot activate. Its effect is thus much more severe than the effect of MASP2*p120G, another deficiency-causing variant that disrupts binding to PRMs since it probably reduces binding opportunities with full-working MASP-2 homodimers in heterozygotes (reviewed in 60). However, not all associations are straightforward. Some of the investigated variants' pleiotropism, whose eQTLs mostly associate with neighboring protein-coding and lncRNA genes, cannot be overestimated. This may underly the opposite association results with *MASP2*2B1-i*: resistance with leprosy *per se* (as previously reported by (30) and susceptibility to HBV infection *per se*. This haplotype is related to intermediate, fully functional MASP-2 serum levels.

Both investigated complement receptor genes presented polymorphisms associated with HBV infection. Like the former genetic associations with higher susceptibility to HBV infection, probably due to genetically determined lower expression and structural protein defects, the p.1208Arg variant (rs2274567) within all *CR1*3A* haplotypes may alter the function of this complement receptor. However, it is still unclear if it will hinder the recognition of C3b deposited on opsonized pathogens. In agreement with our results, heterozygosity for the p.1208Arg was associated with an increased risk for HBV-related liver disease in younger men, drinkers, and non-smokers from the Guangxi Chinese population. Women with the *rs3811381*G* allele and heterozygous for p.1208Arg had a reduced risk of HBV-related liver cirrhosis (64).

Despite being the least studied among the genes chosen for this analysis, *VSIG4* was previously investigated for expression in HBV-infected patients. Also named as Z39Ig or CRIg, *VSIG4* encodes a macrophage complement receptor and negative regulator of T cell activation (65), whose expression is down-regulated by IFN-gamma in macrophages of patients with chronic HBV infection. Its expression is positively correlated with viral load and inversely with serum alanine aminotransaminase levels. The authors also suggested that T cells probably maintain their anti-viral function (secreting IFN-γ) in chronic HBV infection by down-regulating the expression of this co-inhibitory molecule (66). This gene is highly conserved, and our study is the first to investigate its polymorphisms in a candidate-gene approach. In our study, the *VSIG4*TGGRCG* haplotype was associated with increased susceptibility to HBV infection although none of the X-linked *VSIG4* eQTL variants within this haplotype seems associated with altered *VSIG4* gene expression/structure (GTEx Portal). The opposite association between two relatively common *VSIG4* haplotypes that differ in three polymorphic positions—*TGARTA* (protective) and *TGGRCG* (susceptibility) seem to rely chiefly on the two last polymorphisms, which occur within an enhancer region (data from the ENCODE project, UCSC Genome Browser).

This is the most thorough and complete investigation done with genetic polymorphisms of complement components and HBV infection. However, our work does not compare with others who evaluated the role of some of these components on HBV chronification and severe disease since most of the leprosy patients already resolved viral infection at the time of sampling. Furthermore, patient samples were collected in 2002. During that year, HBV detection by real-time quantitative PCR started to get recommended as a diagnostic and prognostic tool in the literature [reviewed by (67)] but was still not established for clinical practice. In our setting, HBV quantification would have been of value for five individuals with active HBV infection, as well as for the detection of a possible occult HBV infection (68). In practice, this would nevertheless leave our results unchanged since we checked for associations with HBV infection *per se*, not active/past acute/chronic infection or its complications.

Moreover, the almost complete absence of current HBV infection (determined by positive HBsAg) among this study's participants is also the most plausible reason for the lack of association with serum levels of the investigated soluble proteins. Thus, the presence of HBV positive serum markers was not accompanied by an inflammatory response at sampling time. Furthermore, the fact that the evaluated *C3* polymorphisms were not associated in this setting does not exclude its importance in the anti-viral immunological response, as mentioned before. Sample size, especially in the lepromatous and non-lepromatous groups, limited the statistical power of this study. Thus, exclusive results from these groups should be cautiously evaluated. Finally, although we found substantial support for functional causality of the associated polymorphisms and gene expression/structure, studies confirming their role in the modulation of HBV infection and HBV/leprosy coinfection are still missing, and the reported associations must be replicated in other settings, to be confirmed.

This work is also unique in the retrospective approach of comparing leprosy patients who have had/had not HBV infection and identifying possible genetic variants whose effects manifest particularly within the leprosy-affected group by comparing the results with those obtained with HBV positive patients and blood donors. Furthermore, the associations found for HBV infection or leprosy/HBV infection were, with few exceptions, not the same as formerly found for leprosy *per se*, agreeing with different roles probably played by complement on the routes used by HBV and *M. leprae* for successful infection and to induce disease (25–31). Finally, our results lead us to suggest that HBV infection occurs more often in individuals carrying common variants that affect protein structure or induce

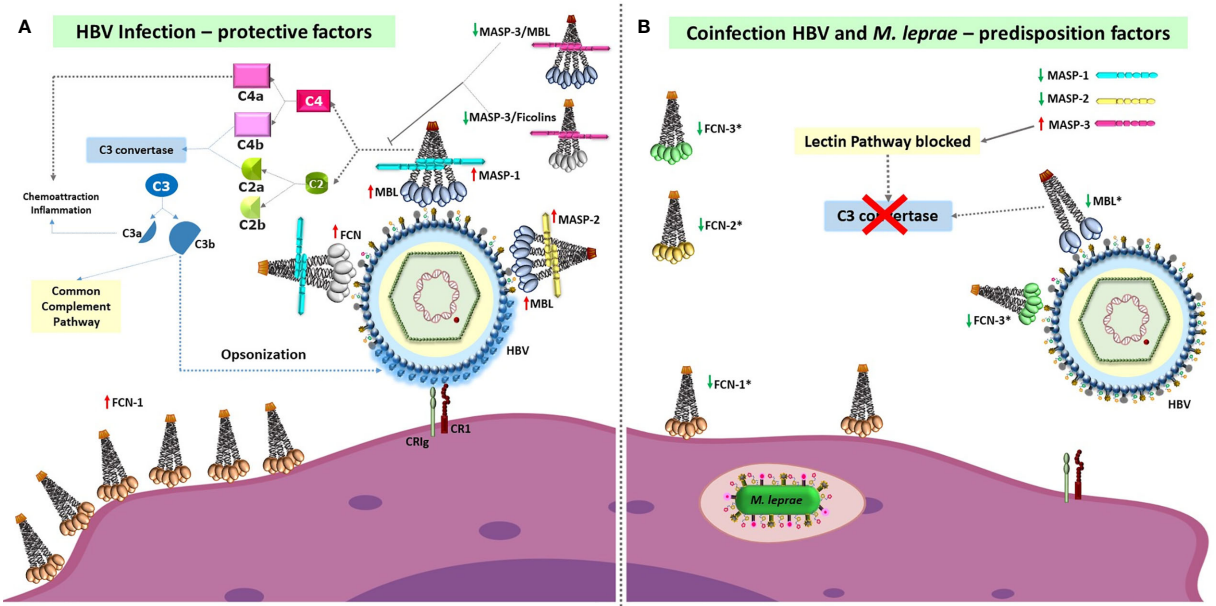

FIGURE 1 | Susceptibility and protective factors of the lectin pathway of complement and complement receptors, for HBV infection *per se* and HBV infection/leprosy. **(A)** High levels of fully functional proteins from the lectin pathway (with the possible exception of MASP-3), and probably also of complement receptors (CR1 and CRIg, officially VSIG4), are necessary to defeat HBV infection through complement activation and opsonophagocytosis. Recognition of HBV-associated molecular patterns by MBL and FCNs associated with MASPs causes MASP-1 autoactivation, MASP-1-driven transactivation of MASP-2 (complexed to another MBL or FCN oligomer), cleavage of C4 and C2, formation of the C3 convertase and cleavage of C3, covering the virus with C3b opsonins. These are recognized by CR1 and CRIg complement receptors, leading to phagocytosis and viral destruction. FCN-1 may also be presented on the phagocyte cell surface through recognition of self-sialic acid residues, but its function as a membrane receptor remains elusive. **(B)** In contrast, low levels of pattern-recognition receptors of the lectin pathway (marked with an asterisk), as well as of other complement proteins, are associated with higher susceptibility to HBV infection and leprosy/HBV infection (represented by *Mycobacterium leprae* within the phagolysosome). The associated polymorphisms cause: dysfunctional MBL, FCN-3 and MASP-2 molecules (given by the p.57E "C" variant in MBL, the frameshift caused by the *+1637* deletion in FCN-3, and p.439H variant in MASP-2), lower MASP-1 levels and higher MASP-3 levels (associated with the *MASP1*AC_CC* haplotype), lower FCN-2 serum levels due to preferential alternative splicing of exon 2 and production of higher amounts of FCN-2 proteins with a shorter collagenous tail (associated with the *FCN2*GGGCAC* haplotype), and lower levels of functional FCN-1 levels (associated with eQTLs of the *FCN1*1* haplotype). These alterations are expected to greatly reduce activation of the lectin complement pathway or to inhibit it (*e.g.*, due to higher MASP-3 levels, as well as dysfunctional MASP-2 molecules). Furthermore, the p.1208Arg encoded by *CR1*3A* haplotypes and CRIg molecules encoded by *VSIG4*TGGRCG* increase susceptibility to the disease, probably by affecting internalization of the virus. MBL, Mannan-binding lectin; FCN, ficolin; MASP, MBL-associated serine protease; CR1, complement receptor 1; CRIg, complement receptor immunoglobulin, encoded by VSIG4 (V-set and immunoglobulin domain containing 4). Red upward-pointing arrows: high/normal expression of functional MASP homodimers/MBL/FCN oligomers. Green downward-pointing arrows: low expression of functional MASP homodimers/MBL/FCN oligomers (sometimes accompanied by high levels of dysfunctional molecules, forming dimers/trimers that do not complex with MASP molecules).

low levels of LP components, decreasing the activity of the proteolytic cascade and possibly phagocytic activity (**Figure 1**). Furthermore, ficolin gene polymorphisms represent another layer of genetic predisposition, modulating the susceptibility to HBV infection in leprosy patients. The associations revealed in this setting lead us to suggest a critical role of the LP and complement receptors in controlling susceptibility to HBV infection, an association maintained and even reinforced within the context of leprosy disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical ethics committee of the HC-UFPR. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IM administered the project and supervised this work. AB contributed to the conception of the work, and curated and analyzed the data. AM obtained and prepared the samples. IM, AB, HM, GK, STS, FA, VB, and LG performed the investigation. AB, HM, SS, FA, LG, and GK further provided methodological

input by developing the multiplex PCR-SSP methods for genotyping. ES provided the samples, and IM, TV, and ST provided resources for the analysis. IM, ST, and TV acquired the funding. AB and CO drafted and edited the manuscript, after critical review for intellectual content, by all coauthors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.574457/full#supplementary-material

Supplementary Figure 1 | Distribution of serum levels of complement components in HBV+ (white) and HBV– (gray) leprosy patients. Sample sizes for each measured component are listed in **Table 2**. Box-whisker plots depict 10th, 25th, 50th, 75th and 90th percentiles.

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