REGULATION OF IMMUNITY TO PARASITIC INFECTIONS ENDEMIC TO AFRICA

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REGULATION OF IMMUNITY TO PARASITIC INFECTIONS ENDEMIC TO AFRICA

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Editorial: Regulation of Immunity to Parasitic Infections Endemic to Africa

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Keywords: parasitic infections, immune response, malaria, trypanosomiasis, leishmaniasis, toxoplasmosis, fascioliasis

Editorial on the Research Topic

Regulation of Immunity to Parasitic Infections Endemic to Africa

Parasitic diseases that affect both humans and animals are major causes of morbidity and mortality across the world, and particularly in Africa where they are endemic (1). They are often closely related to poverty and with the exception of malaria, are considered neglected because they receive relatively lower treatment and research funding in comparison to HIV/AIDS and tuberculosis (2). However, their combined socioeconomic impact in sub-Saharan Africa is comparable to that of tuberculosis and HIV/AIDS (3). Parasitic infections can be transmitted across geographical barriers and warrant global attention (4). Environmental factors such as humidity and warm temperature promote year-round development of parasites and insect vectors, thereby sustaining transmission. In addition, poor sanitary living conditions and overcrowding promote disease transmission.

A better understanding of parasite biology, pathology, immunology, and parasite-host interactions has resulted in better therapeutics and management strategies that have significantly improved patient outcomes. Unfortunately, many parasites develop resistance (5) thereby thwarting the effectiveness of therapeutic strategies. Parasite genomes encode diverse proteins that interact with the host immune system in a dynamic and complex fashion that leads to evasion of protective anti-parasitic mechanisms (6). The articles published in this Research Topic provide insights on current advances in research on parasite biology, host immune responses to parasites and novel therapeutic strategies for parasitic infections such as malaria, trypanosomiasis, leishmaniasis, toxoplasmosis, and fascioliasis.

Several original articles in this Research Topic focus on antigen-specific immune responses to the malaria parasite. Aniweh et al. demonstrated that antibodies against the newly characterized *Plasmodium falciparum* merozoite associated protein (PfMAAP) potently prevented the infection of red blood cells by *P. falciparum* merozoites and were associated with a reduced risk of malaria in humans. Kivisi et al. showed that maternal-derived antibodies against variant surface antigens of *P. falciparum* imposed a selection pressure on the parasites and was associated with reduced parasitemia in infants. This latter study supports a potentially new mechanism for maternal-induced immunity against malaria in the early stages of infancy. The transmission of *P. falciparum* from mosquitoes to humans can be significantly reduced by targeting the transmission stage (gametocyte) of the parasite and this has energized the search for transmission blocking vaccine (TBV) candidates. Although significant strides have been made against *P. falciparum* infection, TBV development against *P. vivax* has not been adequately explored.

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P. vivax is the leading cause of malaria in Latin America. To investigate the potential of TBVs against *P. vivax*, Tentokam et al. analyzed the seroprevalence of antibodies against Pvs230D1M in naturally infected individuals in Brazil and Cambodia. This antigen is located in gametes of *P. vivax* and few polymorphisms have been reported worldwide. They detected similar levels of antibody responses to Pvs230 in these distinct populations from regions with differing transmission intensities, highlighting its potential as a TBV. A separate study focused on the significant role of T-cells in immunity against malaria. Frimpong et al. compared the expression of markers of T-cell inhibition and senescence in healthy children to those with symptomatic or asymptomatic malaria, and have made striking differences observations. Children with symptomatic malaria expressed higher levels of inhibitory and senescent markers on their T cells compared to asymptomatic patients and healthy controls. This suggests that effector T cell function may be impaired in patients with symptomatic malaria and could result in elevated parasitemia.

Parasites belonging to the genus *Trypanosoma* are transmitted by the tsetse fly and cause "sleeping sickness" in humans and wasting disease in livestock. Although the disease currently affects thousands of people and millions more are at risk, trypanosomiasis in animals is more prevalent and poses serious agricultural and economic problems in affected regions. The production of proinflammatory cytokines by macrophages is essential for resistance. However, trypanosome-induced intracellular signaling pathways that lead to macrophage activation and production of proinflammatory cytokines remain poorly defined.

Kuriakose et al. showed that the production of proinflammatory cytokines (IL-6, IL-12, and tumor necrosis factor alpha, TNF-α) by macrophages during *Trypanosoma congolense* infection involves the activation of mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) signaling pathways. They further showed that toll-like receptor 2 (TLR2) and the adaptor molecule, myeloid differentiation primary response 88 (MyD88), are critically involved in this process and that deficiency of MyD88 and TLR2 leads to impaired cytokine production and acute death of *T. congolense*-infected compared to resistant mice. This study expands our knowledge about the signaling pathways involved in the immune response to *T. congolense*, a major etiologic agent of African trypanosomiasis in livestock. Campbell et al. proposed a potential mechanism of immune evasion by *T. brucei,* another species of African trypanosomes. They demonstrated that aromatic ketoacids secreted by *T. brucei brucei* promote the expression of heme oxygenase (a stress protein) and inhibit the production of proinflammatory cytokines in macrophages and glia cells. Morenikeji et al. utilized an *in-silico* approach to identify conserved miRNAs that regulate the expression of genes involved in the immune response during bovine *Trypanosoma* infection. They proposed the use of miRNAs as biomarkers for diagnosis, drug design, targeting and treatment of bovine trypanosomiasis.

Leishmaniasis is endemic in North, East and Central African countries. Globally, nearly 30,000 deaths occur annually and over 1 billion people are at risk of infection. The production of proinflammatory cytokines and T-helper cell responses both play a major role in immunity to *Leishmania* infection. Münck et al. showed that during the early stages of *Leishmania major* infection, the transcription factor, aryl hydrocarbon receptor (AhR), was significantly upregulated in murine lesion-associated macrophages and was associated with increased production of proinflammatory cytokines such as tumor necrosis factor (TNF). The local administration of an AhR agonist to susceptible BALB/c mice resulted in reduced disease severity as well as a decreased Th2 response and parasite burden, suggesting a critical role of this pathway in resistance. McFarlane et al. showed that in contrast to findings in BALB/c mice possessing a global knock out of IL-4Rα, deficiency of IL-4Rα on either $CD4^+$ T cell or total T cells in BALB/c mice did not significantly affect their resistance to *L. donovani* infection. This study suggests that the observed protective role of IL-4 and IL-13 in *L. donovani*[1] infected IL-4Rα global knockout mice (7, 8) is not mediated by IL-4Rα-responsive T cells.

Other parasitic infections highlighted in this Research Topic include fascioliasis and toxoplasmosis. Chen et al. examined the role of Cathepsin B, a lysosomal protease of *Fasciola* and assessed its effects on peripheral blood mononuclear cells (PBMCs) derived from goats. They demonstrated that recombinant[2] *Fasciola gigantica* cathepsin B (rFgCatB) protein decreases the viability of PBMCs *in vitro* but increases their expression of nitric oxide and cytokines such as IL-4, IL-2, IL-10, IFN-γ, TGF-β, and IL-17. He et al. investigated the changes in gene expression of porcine tissues following *Toxoplasma gondii* infection. In addition to tissue-specific transcriptional changes, they observed an elevation in the expression of genes related to the immune response, while the expression of genes involved in metabolic pathways such as lipid metabolism was reduced.

This Research Topic also includes review articles on immune responses to malaria and trypanosomiasis and the role of macrophage migration inhibitory factor (MIF) on the immune response to parasitic infections. Muthui et al. conducted a systematic review of studies in African populations which examined the antibody[3] response to Pfs230 and Pfs48/45 antigens, expressed by *Plasmodium* gametocytes. Although antibodies to both antigens were detected in most studies reviewed, there was significant heterogeneity between study results due to different methods used. This underscores the importance of standardized protocols for conducting scientific studies. Kimenyi et al. critically reviewed the dynamics of host-parasite immune interactions in patients with asymptomatic malaria and proposed the use of RNA sequencing to investigate the immune response during asymptomatic malaria infection.

[1]Vector-borne Diseases. Available online at: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases (accessed March 12, 2020).

[2]WHO. *Environmental Factors Influencing the Spread of Communicable Diseases. WHO* Available online at: https://www.who.int/environmental_health_emergencies/disease_outbreaks/communicable_diseases/en/ (accessed March 21, 2020).

[3]gohrd_analysis_leishmaniasis.pdf. Available online at: https://www.who.int/research-observatory/analyses/gohrd_analysis_leishmaniasis.pdf (accessed April 4, 2020).

In their review, Onyilagha and Uzonna thoroughly examined the factors that affect the immune response to African trypanosomiasis and discussed several immune evasion strategies adopted by this parasite. In particular, they focused on factors that regulate immunity and immunosuppression during *T. congolense* infection and highlighted the possibility that these immunosuppressive factors could aid the evasion of host immune defenses by the parasites.

Ghosh et al. reviewed the effect of macrophage migration inhibitory factor (MIF) secreted by parasites on the host immune response to parasitic infections. The authors also highlighted the potential of MIF as a therapeutic target against parasitic infections.

Collectively, the articles in this Research Topic highlight the complexities of parasite-host interactions, immune responses and disease pathology in parasitic infections. In addition to a better mechanistic understanding of the biology and pathogenesis of parasitic diseases, insights obtained from the articles published in this special issue may contribute to the development of more targeted and effective strategies to control parasitic infections. Importantly, therapeutic agents such as antibodies to *Plasmodium* antigens which promote beneficial host immune responses against parasites may serve as potential treatment options to control disease symptoms and severity. Thus, this is a much-needed and important area of research to increase the armory of tools against parasitic diseases endemic to Africa.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Phenotypic Evidence of T Cell Exhaustion and Senescence During Symptomatic *Plasmodium falciparum* Malaria

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T cells play significant roles during *Plasmodium falciparum* infections. Their regulation of the immune response in symptomatic children with malaria has been deemed necessary to prevent immune associated pathology. In this study, we phenotypically characterized the expression of T cell inhibitory(PD-1, CTLA-4) and senescent markers (CD28(-), CD57) from children with symptomatic malaria, asymptomatic malaria and healthy controls using flow cytometry. We observed increased expression of T cell exhaustion and senescence markers in the symptomatic children compared to the asymptomatic and healthy controls. T cell senescence markers were more highly expressed on CD8 T cells than on CD4 T cells. Asymptomatically infected children had comparable levels of these markers with healthy controls except for CD8+ PD-1+ T cells which were significantly elevated in the asymptomatic children. Also, using multivariate regression analysis, CTLA-4 was the only marker that could predict parasitaemia level. The results suggest that the upregulation of immune exhaustion and senescence markers during symptomatic malaria may affect the effector function of T cells leading to inefficient clearance of parasites, hence the inability to develop sterile immunity to malaria.

Keywords: malaria, *Plasmodium falciparum*, T-cell, exhaustion, immune senescence, PD-1, CTLA-4, CD57

BACKGROUND

Clinical malaria is a disease of public health importance due to its associated morbidity and mortality (1). With the emergence of drug-resistant parasites and insecticide resistant vectors, there is a need to develop effective interventions (2–4). Despite promising results of candidate vaccines in naïve individuals, comparatively poorer responses are observed in people in endemic areas (5, 6), indicating that much effort needs to be focused on understanding host factors associated with the development of immunity, especially in malaria-endemic areas. Blood stage infection with malaria parasites may either result in asymptomatic malaria, uncomplicated malaria, or proceed to complications such as severe malaria anemia or cerebral malaria. Repeated exposure to parasites usually results in the acquisition of anti-disease immunity which is characterized by the absence of

clinical symptoms, yet with susceptibility to the infection. This suggests that the naturally induced immune response generated against *P. falciparum* may not always be potent enough to eradicate the infection. Therefore, malaria vaccines that can protect against symptomatic disease and possibly also eliminate infections remain a global health priority.

Lymphocytes, including T cells, play a significant role in the generation of protective malaria-specific responses (7), and their mechanism of action may either be by controlling or decreasing parasitemia (8) or by exacerbating the infection promoting parasitemia (9). However, looking at natural infections it can be presumed that the inability to eliminate *P. falciparum* malaria may be associated with immune dysfunction resulting from the expression of markers that negatively regulate T cell activity or result in their ineffective response. These may lead to the exhaustion of T cells, which has been well-described in viral infections including HIV and hepatitis B (HBV) (10, 11) as well as in protozoan infections like Toxoplasmosis and Leishmania (12, 13).

In malaria, work in both human and murine models has reported the upregulation of immune inhibitory markers such as T-cell immunoglobin and mucin domain-3 (TIM-3), lymphocyte-activation gene-3 (LAG-3) and programmed cell death-1 (PD-1) during acute infections (14, 15). These have been shown to affect not only the effector functions of T cells including cell proliferation and cytokine production but also antibody generation by B cells (16). Specifically, PD-1 has been associated with decreased cytokine production and proliferation in T cells as well as enhancing disease progression, whereas CTLA-4 has been associated with T cell anergy and establishment of immunological tolerance (17, 18). Furthermore, it has been shown that the dysfunctional nature of exhausted T cells in murine models of malaria can be reversed by blockage of these receptors as this enhances effective parasite clearance and acquisition of immunity (16, 19).

In addition to immune exhaustion, infectious pathogens such as Cytomegalovirus (CMV) and Human Immune deficiency virus (HIV) have been associated with accelerated aging of the body's immune defense system through the upregulation of CD57, a classical marker for immune senescence (20, 21). CD57 is a terminally differentiated marker found on some cell subsets including T cells (22–24). Naïve T cells express CD28, a co-stimulatory molecule that provides signaling for T cell activation) after antigen recognition and this may bind to B7 proteins to provide co-stimulatory signals (25, 26). However, repeated T cell activation is associated with the progressive loss of CD28, a characteristic of memory or terminally differentiated cells, and the corresponding upregulation of CD57 (27–29). These senescent cells are characterized by shortened telomeres, replicative senescence, loss of CD27 resulting in a low proliferative capacity of the cells (30), eventually, leading to an inability to eradicate an infection. Importantly, the expression of CD57 is associated with repeated antigen stimulation (31) which was identified to accurately predict replicative senescence (22). In addition, CD57 expression on CD28- T cells has been shown to differ from the normal aging T cell phenotypes (CD28-CD57+, similarly observed in CMV) (31, 32) found in HIV infections (33).

Cellular aging has been described in wild birds chronically infected with malaria (34). Interestingly, a recent study reported evidence of cellular aging in travelers with single acute *P. falciparum* infections, characterized by decreased telomerase activity and increased levels of CDKN2A, a molecular marker associated with cellular aging (35). Nevertheless, it remains to be elucidated if frequent exposure to malaria is associated with increased expression of markers of T cell senescence in endemic areas. Here, we determined the expression profile of inhibitory or exhaustive, and immune-senescence markers on both CD4+ and CD8+ T cells. We characterized the expression of PD-1, CTLA-4, CD28 and CD57 markers in children with symptomatic malaria, asymptomatic malaria and healthy controls. In addition, we also determined the impact of these T cell phenotypes on parasitaemia and inflammation (using the platelet-to-lymphocyte ratio).

MATERIALS AND METHODS

Ethics Statement

The study protocols were approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research at the University of Ghana. All participants were children and informed consent was obtained from parents or guardians and assent properly received from the children before they were enrolled in the study. All methods were performed in accordance with the relevant guidelines and regulations.

Study Subjects

A total of 57 children within the age ranges of 1–12 years were recruited for the study, consisting of healthy children with no *P. falciparum* infections ($n = 17$), children with asymptomatic *P. falciparum* malaria ($n = 18$) and children with clinical malaria ($n = 22$) who were recruited from the Asutsuare and the Paakro sub-districts which are hyper-endemic areas for malaria transmission in Ghana. A volume of 5 ml of venous blood was collected from all study participants after recruitment. Parasites were identified using Giemsa stained thick and thin blood films. Clinical cases of malaria recruited from the health centers were defined by a history of fever within 24 h of health center attendance and presence of parasitaemia. For clinical cases, we collected venous blood samples from the children before anti-malarial treatment, based on the nationally recommended guidelines. Asymptomatic cases were recruited from the community and were defined by the presence of parasitaemia, absence of fever and no signs or symptoms of the disease. Healthy children, also recruited from the community were selected based on the absence of parasitaemia, fever and no signs or symptoms of the disease.

Peripheral Blood Mononuclear Cells (PBMC) and Plasma Isolation

Isolation of PBMCs was performed by density gradient centrifugation using ficoll paque. After isolation, PBMCs were enumerated and cryopreserved in fetal bovine serum with 10%

dimethyl sulfoxide. PBMCs were kept at −80°C overnight and subsequently transferred to liquid nitrogen until required for the experiment.

Flow Cytometry Analysis

PBMCs were retrieved, thawed and washed. The viability was measured by the trypan blue dye exclusion method and cells with viability > 95% were used in the assay. All the antibodies were purchased from BD except anti-human FOXP3 fluorochrome-conjugated antibody (Biolegend). After washing, the cells were extracellularly stained with the following antibodies: anti-CD3-(APC H7), anti-CD4 (BUV 395), anti-CD8 (PerCP Cy5.5), anti-CD28 (APCR700), anti-CD57 (FITC) and anti-PD-1 (BUV737) on ice for 30 min. The cells were washed, fixed and permeabilized using FOXP3 buffer set (BD) according to manufacturer's instructions and intracellularly stained for FOXP3 (PE) and CTLA-4 (APC) on ice for 40 min. We gated for T cells by CD3, CD4, and CD8 lineage markers. Gates for inhibitory and senescence markers were defined using fluorescence minus one controls (**Figure S1**). Cells were acquired on a BD LSR Fortessa II-X20 cytometer. Data were compensated and analyzed using Flowjo V10 software (Tree Star, San Carlos, CA).

Statistical Analysis

Data analyses were performed with R-studio for statistical analysis (version 2) and the GraphPad Prism version 6.01 (GraphPad Software, Inc.). For comparing the markers of T cells among the three study populations, the Kruskal-Wallis test with a Dunn's *post hoc* test for multiple comparisons was used. Spearman's rank correlation was used to determine associations between markers. Principal component analysis (PCA) was conducted to identify and visualize significant features of T cell phenotypes (degree of variation) that can cluster our study populations by considering all phenotypes measured. PCA which is an unsupervised learning algorithm provides dimensions (linear combinations) along which the data are separable and reduces the noise associated with data whilst increasing its robustness. PCA was used since it reduces the data set to a small set of patterns and retain the significant features that are responsible for variation (separating the data into clusters). Multiple linear regression models with likelihood ratio test were also used to investigate the association between parasitaemia or inflammation and the measured cellular markers. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical Characteristics of the Study Participants

The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board. This was a cross-sectional study in which we recruited 57 children in the age range of 1–12 years. The participants included 22 symptomatic children, 18 asymptomatic children and 17 healthy controls (**Table 1**). The sexes of the children were comparable amongst the study groups ($p < 0.05$). Healthy children were older than the asymptomatic($p < 0.05$) and symptomatic children ($p < 0.05$). Levels of parasitemia mirrored the intensity of infection, with symptomatic children having a higher parasite load compared to the asymptomatic children ($p < 0.001$). Hemoglobin levels were significantly decreased in the symptomatic children in comparison to the asymptomatic children ($p < 0.05$). Even though, the lymphocyte count was not significantly different amongst the study groups, they also mirrored the intensity of infection (p>0.05). We found the granulocyte count to be comparable amongst the study groups ($p > 0.05$). Also, the platelet-to-lymphocyte ratio (PLR) was found to be comparable between the healthy controls and asymptomatic groups ($p < 0.05$) but higher than the symptomatic group ($p < 0.05$).

Increased Expression of PD-1 and CTLA-4 Markers on T Cells in Children With Symptomatic *P. falciparum* Malaria

We first investigated the expression of the inhibitory markers PD-1 and CTLA-4 on T cells **(Figure 1A)**. The expression levels of PD-1 were significantly upregulated in the symptomatic children compared to the asymptomatic ($p < 0.0001$) and healthy groups ($p < 0.0001$) for the CD4+ T cells (**Figure 1B**). Levels of PD-1 in the asymptomatic children and healthy children were comparable. Similarly, CD8+PD-1+ T cells were upregulated in children with symptomatic malaria compared to asymptomatic ($p = 0.0312$) and uninfected controls ($p < 0.0001$). Nevertheless, the expression of PD-1 on CD8+ T cells was increased significantly in the asymptomatic children compared to the healthy controls ($p = 0.0359$). Of note, the levels of PD-1 were higher in CD8+ T cells compared to the CD4+ T cells in all study groups. Also, the expression levels of CTLA-4 on CD4+ T cells were increased significantly in the symptomatic children compared to the asymptomatic ($p < 0.001$) and healthy controls ($p < 0.05$) whereas comparable levels of expression were found between asymptomatic children and healthy controls (**Figure 1C**). This trend was the same for the levels of CTLA-4 expression on CD8+ T cells among the study groups: symptomatic children had increased levels compared to asymptomatic ($p < 0.001$) and healthy children ($p < 0.05$).

Next, we assessed the expression of PD-1 and CTLA-4 double-positive markers on both T cell subsets. The symptomatic children had significantly higher levels of PD-1 and CTLA-4 double positive markers on CD4+ T cells in comparison to the asymptomatic children ($p < 0.0001$) and healthy controls ($p = 0.0091$). Similarly, levels on CD8+ T cells were higher in symptomatic children compared to the asymptomatic children ($p = 0.0121$) and healthy controls ($p = 0.0098$) (**Figure 1D**). In all, levels of PD-1 and CTLA-4 double positive markers between the asymptomatic children and healthy controls were comparable and not significantly different.

The significant levels of inhibitory markers observed in children with symptomatic malaria may be related to the inadequacy of effector functions in clearing parasitemia.

TABLE 1 | Clinical characteristics of the study participants.

Characteristics	Control (C)	Asymptomatic (A)	Symptomatic (S)	*P*-values
Participants	$n = 17$	$n = 18$	$n = 22$	
Age (IQR), years	9(8–11)	7(4.5–9)	6(4.8–7)	0.0087[a]
Female (%)	52.94	44.44	50	0.8765[b]
Hemoglobin, g/dl[#]	11.5(0.994)	12.7(1.234)	10.7(3.025)	0.0402[c]
Parasitemia (IQR), μl	NA	845(260.7–3812)	13973(7238–58764)	0.0009[d]
Granulocytes (10^9/L)[#]	3.353(1.335)	3.069(2.115)	5.041(3.310)	0.0518[c]
Lymphocytes (10^6/L)	2.9(2.5–3.6)	2.1(1.2–3.45)	1.9(1.3–3.9)	0.0889[a]
Platelets (10^9/L)	305(237–356)	223(193–280)	101(61–198)	>0.0001[a]
PLR(IQR)[&]	95.31(82.79–133.9)	70.97(27.52–205.3)	53.54(31.19–95.31)	0.0345[a]

IQR, interquartile range. [a]*Kruskal Wallis test.* [b]*Chi-square test.* [c]*One-way ANOVA.* [d]*Mann-Whitney U-test.* [#]*Mean(Standard deviation).* [&]*(C = 17, A = 16, S = 19).*

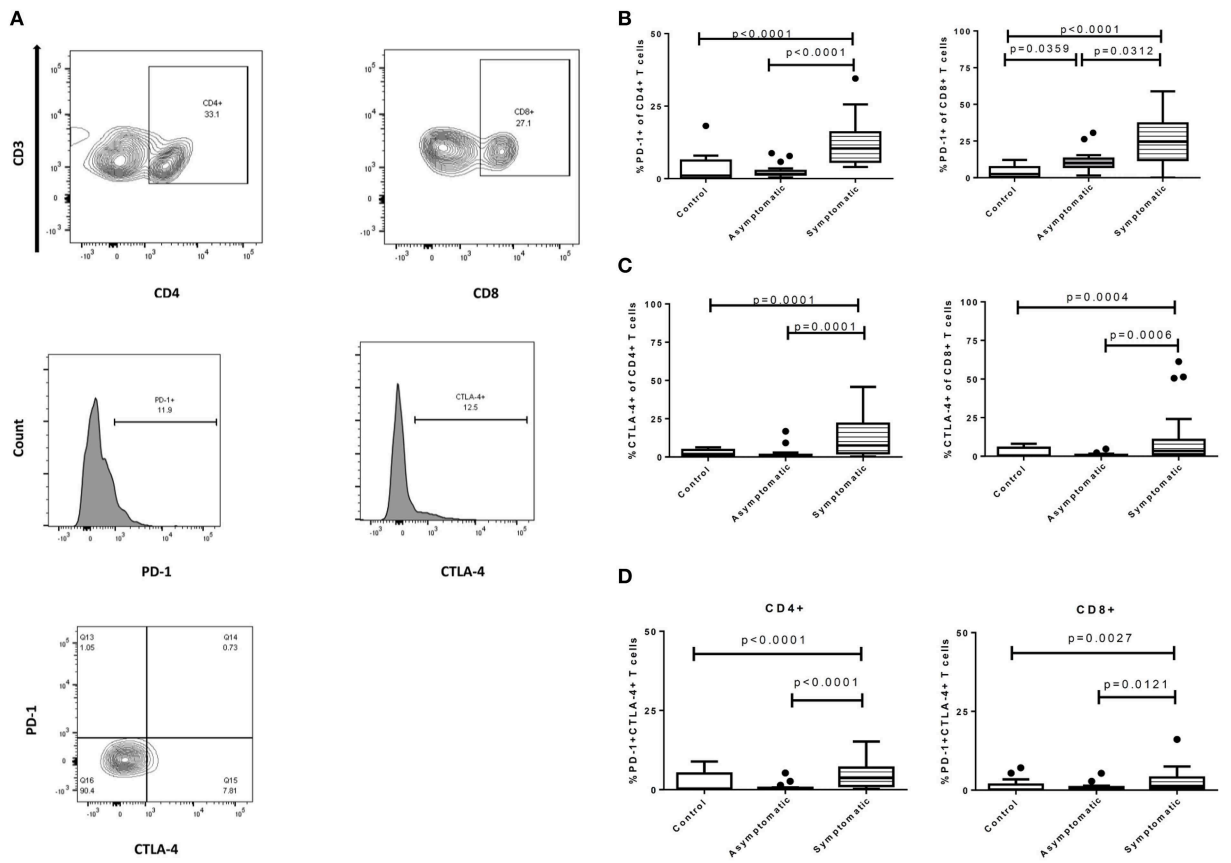

FIGURE 1 | Expression profile of exhaustion and regulatory markers on CD4+ and CD8+ T cells among symptomatic malaria patients ($n = 22$), asymptomatic malaria($n = 18$) and healthy children ($n = 16$). **(A)** The gating strategy to identify expression levels of PD-1 and CTLA-4 markers. The expression profile comparing levels of **(B)**. PD-1, **(C)**. CTLA-4, **(D)**. PD-1 and CTLA-4 double-positive markers, on both CD4+ and CD8+ T cells across the study subjects. Levels of expression were compared using the Kruskal-Wallis with Dunn's test to correct for multiple comparisons. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers with medians indicated by the bold horizontal lines across the boxes. $P < 0.05$ were considered statistically significant.

Symptomatic *P. falciparum* Infection Is Associated With the Upregulation of T-Cell Senescence Markers

We also determined if symptomatic malaria may be associated with the biological aging of T cells, by measuring senescent markers using CD28 and CD57 and comparing the proportions with the asymptomatic and healthy groups. We first determined the proportions of T cells expressing CD57 which were found to higher on CD8+ T cells compared to the CD4+ T cells. This trend was similar in all 3 study groups (**Figure 2**).

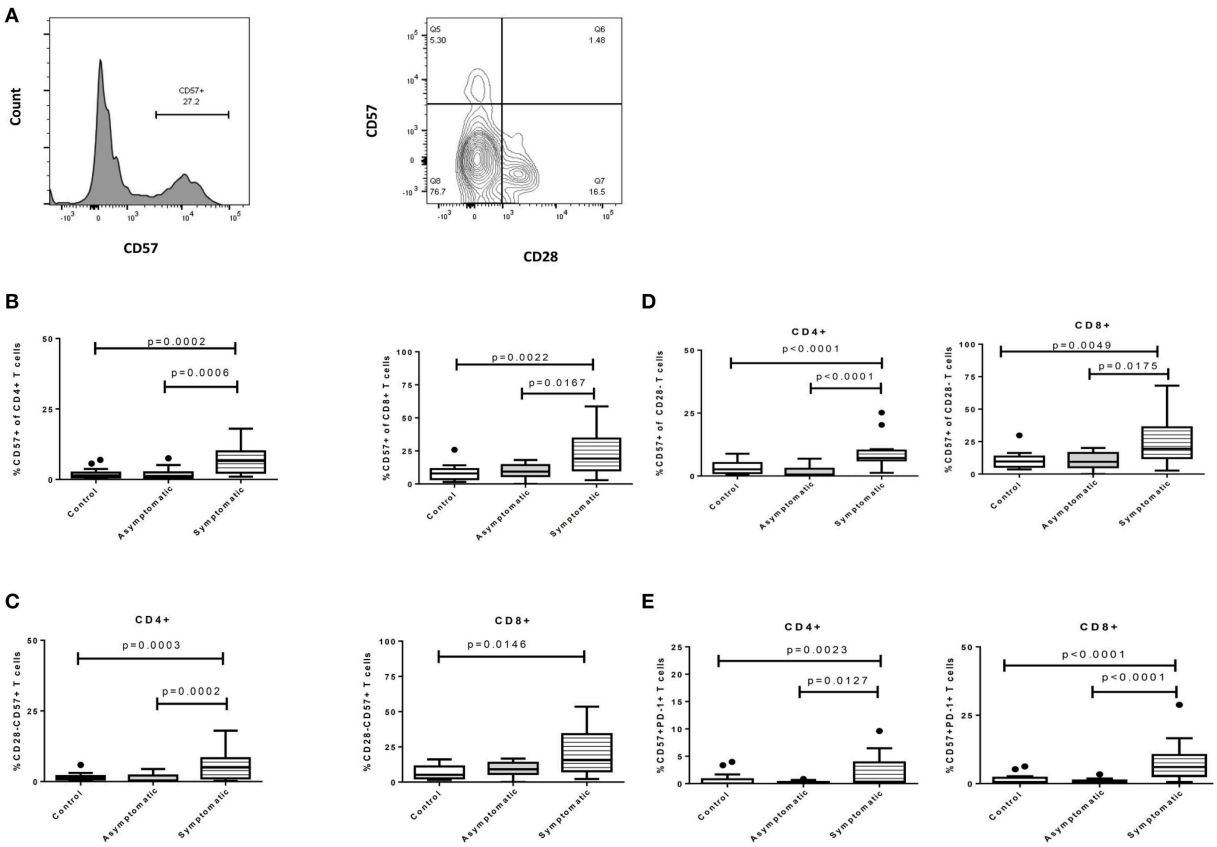

FIGURE 2 | Symptomatic *P. falciparum* infection is associated with the upregulation of T-cell senescence markers. The expression profile of immune senescence markers on CD4+ and CD8+ T cells among symptomatic malaria patients, asymptomatic malaria and healthy children. **(A)** The gating strategy for identifying CD57 and CD28 surface markers. The expression profile comparing levels of **(B)**. CD57, **(C)**. CD28-CD57+, **(D)**. CD57+ of CD28- and **(E)**. PD-1+CD57+ markers on both CD4+ and CD8+ T cells across the study subjects. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers with medians indicated by the bold horizontal lines across the boxes. Levels of expression were compared among the study population using the Kruskal-Wallis with Dunn's test to correct for multiple comparisons. $P < 0.05$ were considered statistically significant.

Levels of CD4+CD57+ T cells were significantly higher in children with symptomatic malaria compared to asymptomatic ($p = 0.0006$) and healthy controls ($p = 0.0041$). A similar trend was observed for the CD8+ T cell subsets where a significant difference was observed between symptomatic and asymptomatic children ($p = 0.0167$) and healthy controls ($p = 0.0050$) (**Figure 2A**). Secondly, we checked for the percentage expression of CD28-CD57+ T cells, a marker frequently associated with T cell aging in the elderly. Levels of CD28-CD57+CD4+ T cells were also increased in children with symptomatic malaria compared to children with asymptomatic infections ($p = 0.0002$) and healthy controls ($p = 0.0064$). In contrast, levels of the CD28-CD57+ marker on CD8+ T cells did not differ between the symptomatic children and asymptomatic children ($p = 0.1115$), but was increased in the symptomatic group compared to healthy controls($p = 0.0178$) (**Figure 2B**).

Later, we gated on CD28- T cells and measured the expression of CD57 on CD28- T cells (CD57 of CD28- T cells) to determine if the expression levels may be similar or differ from what is observed in normal aging or HIV infections. We found that the percentage expression of CD57+ on CD28-CD4+ T cells remained increased in children with symptomatic malaria compared to children with asymptomatic infections ($p < 0.0001$) and healthy controls ($p = 0.0261$). Also, the percentage expression of CD57 on CD28- CD8+ T cells was significantly increased in children with symptomatic malaria compared to those with asymptomatic malaria ($p = 0.0175$) or healthy controls ($p = 0.0147$) (**Figures 2B,C**).

We further compared the expression of CD57 and PD-1 double-positive markers (commonly associated with increased apoptosis) on T cells in the study participants. We observed that CD4+ T cells expressing both CD57 and PD-1 were increased in children with symptomatic malaria compared to asymptomatic ($p = 0.0127$) and healthy controls ($p = 0.0071$; **Figure 2D**). This trend was similarly observed in the CD8+ T cells: levels of PD-1+CD57+CD8+ T cells were increased in children with symptomatic malaria in comparison to asymptomatic children ($p < 0.0001$) and healthy

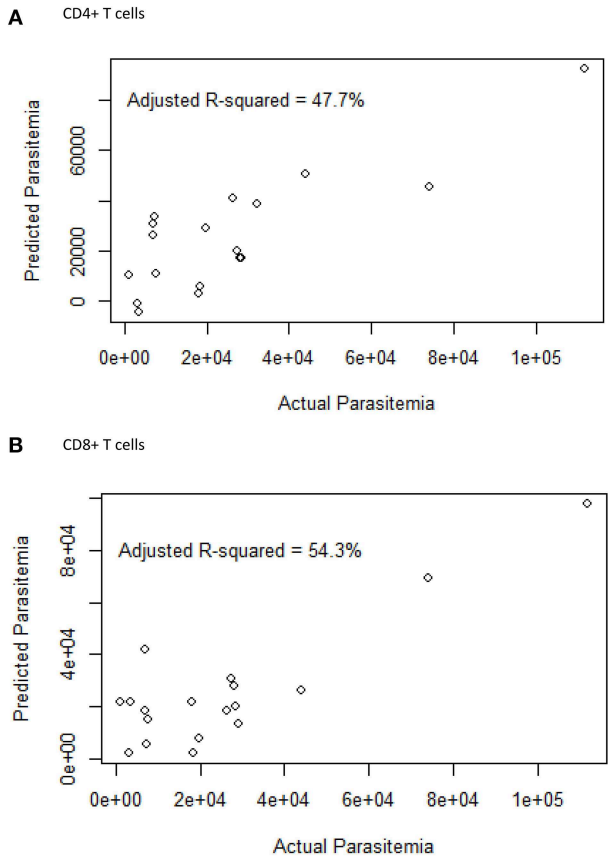

FIGURE 3 | The relationship between the surface markers and parasitemia. A multiple linear regression plot. Actual parasitaemia levels were indicated on the x-axis with the predicted values of parasitaemia on the y-axis. The model was designed using 7 T cell phenotypes measured in **(A)** CD4 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) and **(B)** CD8 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) T cells from PBMCs obtained from the symptomatic children.

controls ($p = 0.0001$). Overall, T cells from symptomatic *P. falciparum* infected children showed phenotypic evidence of T cell senescence.

CTLA-4 Is a Major Predictor of Parasite Load During *Plasmodium falciparum* Infection4

The effect of cellular markers on parasitemia and inflammation was investigated using multivariate regression analysis. We analyzed 7 T cell phenotypic markers; inhibitory (PD-1+, CTLA-4+, PD-1CTLA-4+) and senescence (CD57+, CD28-CD57+, CD57+ of CD28-, PD-1+CD57+)each on both CD4+ and CD8+ T cells to determine if any of these markers could predict parasitaemia or inflammation (PLR). We defined inflammation by the ratio of platelet-to-lymphocyte count (PLR) (36–38). Before the multivariate analysis, we initially performed a correlation analysis to determine if any of the phenotypes may be significantly associated with PLR or parasitaemia. The proportions of PD-1 ($r = -0.65$, $p < 0.01$) and CTLA-4 ($r = -0.506$, $p<0.05$) were inversely correlated with PLR (**Figure S4**) but positively correlated with parasitaemia (for PD-1, $r = 0.4631$, $p < 0.05$; CTLA-4, $r = 0.4831$, $p < 0.05$). However, using the multiple linear regression model and performing a likelihood ratio test, expression levels of CTLA-4 on both CD4+ and CD8+ T cells were found to significantly predict the level of parasitemia in the symptomatic children (**Figure 3**; **Tables 2**, **3**). Likewise, the levels of CD8+CD28-CD57+ and CD57 on CD8+CD28- T cells could significantly explain some of the variation observed in parasitemia (**Tables 2**, **3**). Even though all the coefficients from the regression analysis for the T cell phenotypes were inversely associated with inflammation, none could be a predictor of inflammation (**Table S1**).

On the other hand, among the asymptomatic malaria group, levels of CD4+PD-1+ and CD4+PD-1+CD57+ could predict and explain some of the variation observed in parasitemia ($p < 0.05$; $p < 0.0001$) whereas for CD8+ T cells, the expression of CTLA-4 ($p < 0.001$) and PD-1+CTLA-4+ ($p < 0.0001$) were good predictors of parasitemia (**Figure S3**; **Table S2**).

Multivariate Analysis of T Cell Inhibitory and Senescent Markers

In order to identify significant immunological signatures (T cell phenotypes) that can explain the variation in our study population and separate our study population into clusters, we performed a principal component analysis (PCA). From the eigen values we obtained, we selected principal components that best explained the variations in the datasets. Components 1 and 2 for the CD4+ T cells accounted for 73.1% (62 and 11.7%, respectively) of the variation in data whereas, for CD8+ T cells, PC1 and PC2 accounted for 81.1% (56.3 and 24.8, respectively). From the plots, it can be observed that mostly the symptomatic group had higher PC values compared to the asymptomatic group. Using the entire datasets, the principal components clustered our population into three groups based on the frequencies of the phenotypes (**Figures 4A,B**). Also, from the CD8+ T cells, it can be observed that PC1 is associated with inhibitory markers located in the upper right quadrant whereas PC2 is associated with senescent markers, located in the lower right quadrant. In addition, the loadings of PD-1 and CD57 were significant for PC1 and PC2, respectively. Further analysis indicated the T cell phenotypes contributing to most of the variation for CD4 T cells were CTLA-4 and PD-1 whereas, for CD8 T cells, the important markers were PD-1, CD57 and CTLA4 (**Figure S2**).

The Interrelationship Between the Cellular Inhibitory Markers

Next, we determined the interrelationship between the surface markers (including FOXP3) using partial correlation, a multiparametric correlation analysis that controls for confounding factors (**Tables 4**, **5**). For instance, significant positive correlations for CD4 T cells were observed between

FOXP3 and PD1, FOXP3 and CD57, PD-1CTLA-4 and CD57 ($p < 0.05$). Generally, significant correlations were all positively related.

DISCUSSION

The upregulation of inhibitory and senescent markers on T cells has been associated with the impairment of effector T cell responses. In this study, we sought to identify T-cell immune signatures that may be associated with the development of symptomatic malaria. We analyzed the pattern of expression of co-inhibitory and senescent markers in children with symptomatic *P. falciparum* malaria, asymptomatic malaria and healthy controls. We found that the expression of these exhaustive and senescent markers was increased in children with symptomatic malaria compared to those with asymptomatic infections and healthy controls. Using multivariate regression analysis with likelihood ratio test, we found CTLA-4 to be a strong predictor of parasitemia levels. Also, none of the T cell phenotypes measured was a good predictor of inflammation, even though PD-1 and CTLA-4 were inversely correlated with inflammation. Using a principal component analysis, our study population was clustered into three groups based on the level of expression of the cellular markers. Further analysis revealed that, for CD4+ T cells, the levels of CD4+CTLA4+ and CD4+PD-1+ markers could explain the clustering pattern of the study groups, whereas for CD8+ T cells the important markers were CD8+PD-1+, CD8+CD57+ and CD8+CTLA-4+. In addition, we observed a lower platelet-to-lymphocyte ratio in the symptomatic malaria group probably resulting from the decreased platelets and lymphocytes counts that are associated with clinical malaria (39, 40).

The activation of T cells by pathogens leads to the induction of inhibitory receptors such as CTLA-4 and PD-1 (41). PD-1 and CTLA-4 are some of the well-characterized inhibitory receptors associated with the exhaustion of T cells (16, 42, 43). Levels of expression of the inhibitory markers PD-1 and CTLA-4 were upregulated in children with symptomatic malaria, confirming recent studies that have observed increased levels of PD-1 and CTLA-4 during acute infections (14, 44, 45), resulting in decreased production of cytokines (46). This, therefore, suggests that the increased frequency of inhibitory markers during clinical disease may alter the effector function of T cells.

It has been shown by Butler et al. (16) that levels of CD4+PD-1+ phenotypes correlate with parasitemia in clinical malaria. In this study, we found that CD4+PD-1+ and CD8+CTLA-4+ could also predict parasitemia levels in the asymptomatic malaria group. Importantly, in the symptomatic group, the expression of CTLA-4 was a major predictor in determining parasitemia load. This suggests that T cell exhaustion may induce tolerance which may promote parasitemia. Since both PD-1 and CTLA-4 are negative regulators of the immune response, their observed increase in symptomatic children may indicate that PD-1 and CTLA-4 contribute in regulating T cell activity or inflammation (14, 46). Interestingly, we found an inverse association between these inhibitory markers and inflammation, which is in line with previous observations in murine models that blockage of T cell inhibitory markers exacerbated the immune response, increased susceptibility to severe disease and decreased survival (47, 48). Furthermore, the strong association we observed between T cell exhaustion and clinical parameters such as parasitemia and inflammation suggests that T cell exhaustion plays a vital role in malaria pathogenesis.

We have previously shown that asymptomatic *P. falciparum* infections are characterized by a reduced frequency of regulatory T cells (49). Here, we hypothesized that asymptomatic infections may have reduced expression of inhibitory markers compared to symptomatic children. We found that the expression levels of inhibitory markers in asymptomatic and healthy controls were mostly comparable except for PD-1 which was increased in the *P. falciparum* infected groups compared to the healthy controls (43). It may indicate PD-1 expression may be driven by *P. falciparum* infections. This could also imply that continuous exposure to *P. falciparum* infections may render T cells to be defective in function. Unfortunately,

TABLE 2 | The association of inhibitory markers on CD4+ T cells and parasitaemia for the symptomatic malaria population.

Covariate	Coefficient	*p*-value	LR test (*p*-value)
CTLA4	0.386	0.12	**0.032***
PD1	0.453	0.25	0.11
PD1CTLA4	0.509	0.57	0.44
CD57	0.499	0.48	0.33
CD57–CD28+	0.434	0.2	0.075
PD1CD57	0.52	0.75	0.66
CD28–CD57+	0.503	0.51	0.37

*$p < 0.05$.
Statistically significant values are highlighted in bold.

TABLE 3 | The association of inhibitory markers on CD8+ T cells and parasitaemia for the symptomatic malaria population.

Covariate	Coefficient	*p*–value	LR test (*p*-value)
CTLA4	0.253	**0.018***	**0.0012****
PD1	0.525	0.26	0.12
PD1CTLA4	0.545	0.351	0.2
CD57	0.563	0.491	0.34
CD57+CD28-	0.447	0.098	**0.023***
PD1CD57	0.582	0.792	0.72
CD28-CD57+	0.438	0.09	**0.02***

*Regression coefficients and p-values were determined using a multiple linear regression model. The effect of each phenotype in predicting the degree of inflammation was adjusted using the other considered phenotypes. The likelihood ratio (LR) test was used to assess the significance of each variable's effect by comparing the adjusted model with another model in which the covariate is absent. $P < 0.05$ was considered statistically significant. Statistically significant values are highlighted in bold. * $p < 0.05$, ** $p < 0.01$.*

TABLE 4 | A partial correlation matrix with covariates between the cellular markers measured on CD4+ T cells for the symptomatic malaria population.

CD4	CTLA4	PD1	PD1CTLA4	CD57	CD57+CD28-	PD1CD57	CD28-CD57+	FOXP3
CTLA4	1							
PD1	−0.28392916	1						
PD1CTLA4	0.48244305	**0.7425784****	1					
CD57	−0.09261014	−0.4476809	**0.6287185****	1				
CD57+CD28-	0.28077369	0.1731718	−0.0862313	−0.06116931	1			
PD1CD57	0.36438345	0.1282666	−0.179054	−0.08438154	−0.2904794	1		
CD28-CD57+	0.03186114	0.3141221	−0.456166	**0.91009944****	0.363051	0.1875853	1	
FOXP3	0.2481085	**0.6309751***	−0.4845044	**0.55752428***	−0.432212	−0.0890901	−0.3706132	1

*Significant correlations were determined using a permutation test. P < 0. 05 were considered statistically significant; *p < 0.05, **p < 0.01, ****p < 0.0001. Statistically significant values are highlighted in bold.*

TABLE 5 | A partial correlation matrix with covariates between the cellular markers measured on CD8+ T cells for the symptomatic malaria population.

CD8	CTLA4	PD1	PD1CTLA4	CD57	CD57+CD28-	PD1CD57	CD28-CD57+	FOXP3
CTLA4	1							
PD1	0.4365899	1						
PD1CTLA4	**0.8497852****	−0.267313	1					
CD57	−0.230747	0.0385105	0.136794	1				
CD57+CD28-	0.2025701	0.0277434	−0.1928562	**0.6714082****	1			
PD1CD57	−0.2022586	**0.720932****	0.1076203	0.0563088	0.14287562	1		
CD28-CD57+	0.1714043	−0.4517704	−0.0132945	0.2164709	0.46299502	0.2318686	1	
FOXP3	0.2319811	−0.3906262	0.1694245	0.0678562	−0.00587128	0.3006289	−0.29398143	1

*Significant correlations were determined using a permutation test. P < 0. 05 were considered statistically significant; **p < 0.01, ****p < 0.0001. Statistically significant values are highlighted in bold.*

we could not determine the functionality of these T cells to confirm this.

Immunosenescence is the aging of immune cells characterized by shortened telomeres and inability to replicate, (50) sensitivity to apoptosis and, phenotypically, the expression of CD57 (22). T cell senescent markers were more associated with CD8+ T cells compared to CD4+ T cells consistent with earlier reports that they accumulate at lower frequencies for CD4+ T cells in human periphery (51). Here, we found an increased expression of CD57+T cell subsets in children with symptomatic malaria (52). This may suggest that malaria accelerates the aging of the T cell pool. In addition, the increased expression of CD28-CD57+ marker observed in symptomatic children indicates a greater proportion of effector T cells in symptomatic malaria have a memory phenotype since these cell subsets have been described to be antigen experienced (22, 31, 51). It has previously been shown that PD-1+CD57+CD8+ T cells have increased sensitivity to apoptosis mediated by PD-1 (53). The observed increase in expression of CD57 and PD-1 double-positive markers on CD8+ T cells, therefore, indicates a greater risk of apoptosis of these cells in clinical malaria. Additionally, T cell aging has been well characterized in the elderly population, CMV and HIV infections (21, 30, 33). In contrast to CMV infections which leads to expansion of T cell senescent markers, HIV leads to a decrease in the expression of CD57 on CD28-CD8+ gated T cells, whereas levels of CD8+CD28-CD57+ remains unchanged. In our study, we observed that both the proportion of CD28- T cells that express CD57 were expanded in the symptomatic malaria group, suggesting that T cell aging in falciparum infections is more similar to that observed during CMV infections than in HIV infections. Together, these observed phenotypic changes might reduce the responsiveness of the T cell repertoire to *P. falciparum* antigens resulting in an impaired ability to eliminate parasitemia.

FOXP3 is an immune regulatory marker associated with preventing immunopathology during inflammation. Both FOXP3 and PD-1 have been shown to suppress host immune response. Importantly, *P. falciparum* infections has been reported to cause the induction of PD-1+CTLA4+ T cells that control T cell activity (14). In this study, the positive correlation observed between FOXP3 and PD-1 T cells as well as between PD-1 and PD-1CTLA4 T cells could indicate that these markers play complementary roles in mediating the increasing immune activation that is associated with symptomatic malaria. There are conflicting reports about the role of CD57+ T cells in clinical disease, with some reports describing them as immunosuppressive and others suggesting

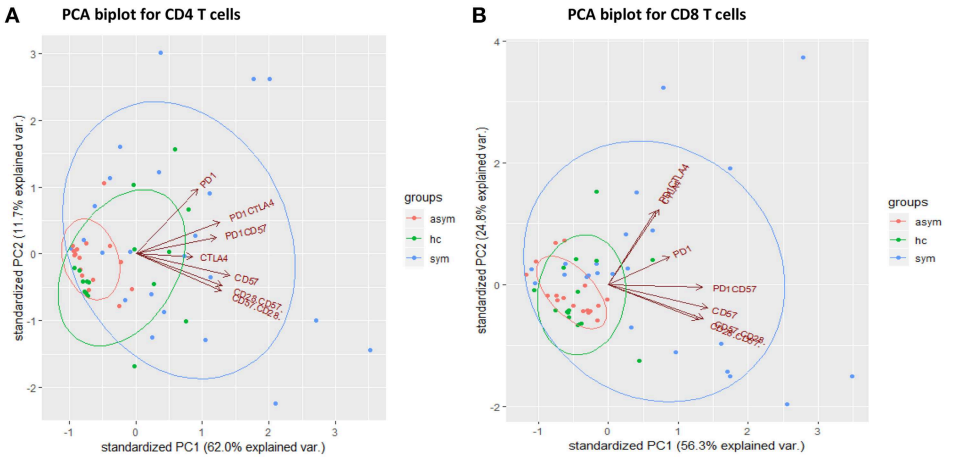

FIGURE 4 | Principal component analysis of cellular markers determined in the healthy groups ($n = 17$), asymptomatic malaria ($n = 18$) and symptomatic malaria ($n = 22$), for **(A)** CD4 and **(B)** CD8 T cells. The green, red and blue symbols as well as ellipses denote the healthy, asymptomatic and symptomatic groups respectively. The red arrows indicate the cellular markers.

they exacerbate immune activity through IFNγproduction (31, 54). Here, we observed a significant positive correlation between CD4+CD57+ T cells and CD4+FOXP3+ T cells as well as between CD4+CD57+ and CD4+PD-1+CTLA-4+ T cell subsets. However, since we could not determine the functionality of the CD57 T cell subsets, we can only suggest that the positive relationship observed between CD4+CD57 and CD4+FOXP3+ as well as CD4+ PD-1+CTLA-4+ T cell subsets may indicate that CD4+CD57+ T cells play suppressive roles during clinical malaria. This further supports the view that *Plasmodium* infections induce immunosuppressive immune responses that enhance the development of tolerance to the parasite, a mechanism affecting the development of sterile immunity.

Also, the results from the principal component analysis may indicate that a selection panel of the considered markers may serve as a biomarker for identifying individuals with symptomatic disease. It may probably be used to predict the outcome or immune response to vaccination. These results provide a basis to perform functional assays to determine the impact of the considered markers on the acquisition of anti-disease immunity during *P. falciparum* infections, preferably in a longitudinal cohort.

Studies have reported a low ratio of platelet-to-lymphocyte count (PLR) as a marker for inflammation in various infectious diseases such as HBV (36) and HCV (37). In this study, even though none of our markers was a good predictor of inflammation as previously stated using the PLR, we show that symptomatic malaria is characterized by low ratio of platelet-to-lymphocyte counts, which is indicative of on-going inflammatory response. Nonetheless, additional studies are needed to determine the significance of other hematological markers of inflammation (such as the neutrophil to lymphocyte ratio) to ascertain their clinical relevance during symptomatic malaria.

This study had a number of limitations due to the cross-sectional nature. We could not determine the effect of anti-malarial treatment on the expression of these inhibitory and senescent markers since samples were taking before the initiation of treatment. Furthermore, we defined *P. falciparum* infections by microscopy which is not able to distinguish between microscopic and sub-microscopic infections. In addition, we could not determine the effect of these markers on T cell cytokine production since cytokine profile analysis was not performed.

Despite these shortcomings, this study shows evidence that the phenotypic defect of T cells during *P. falciparum* infections are more pronounced in clinical malaria and associated with higher expression of exhaustive and senescent markers compared to asymptomatic infections. CTLA-4 was a good predictor of parasitemia in both symptomatic and asymptomatic malaria groups. Also, using the platelet to lymphocyte ratio, none of the markers measured could predict inflammation. In addition, we observed that the aging phenotype of T cells in malaria infection is similar to that observed with normal aging and CMV infections. These may imply that the increased expression of these markers may be associated with the absence of sterile immunity to *P. falciparum* malaria.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Noguchi Memorial Institute for Medical Research, ethical review committee with written informed consent from all subjects. All subjects gave written informed consent and assent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research at the University of Ghana (NMIMR-IRB CPN 096/15-16).

CONSENT FOR PUBLICATION

All authors have read and agreed to the content of this manuscript and its publication upon acceptance.

AUTHOR CONTRIBUTIONS

AF and MO conceived the idea and designed the experiments. WN, MO, and KK supervised the work. AF performed the experiments in the study and was assisted by DA-G and JA. AF, KK, MO and, WN wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01345/full#supplementary-material

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Global Transcriptome Profiling of Multiple Porcine Organs Reveals *Toxoplasma gondii*-Induced Transcriptional Landscapes

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We characterized the porcine tissue transcriptional landscapes that follow *Toxoplasma gondii* infection. RNAs were isolated from liver, spleen, cerebral cortex, lung, and mesenteric lymph nodes (MLNs) of *T. gondii*-infected and uninfected (control) pigs at days 6 and 18 postinfection, and were analyzed using next-generation sequencing (RNA-seq). *T. gondii* altered the expression of 178, 476, 199, 201, and 362 transcripts at 6 dpi and 217, 223, 347, 119, and 161 at 18 dpi in the infected brain, liver, lung, MLNs and spleen, respectively. The differentially expressed transcripts (DETs) were grouped into five expression patterns and 10 sub-clusters. Gene Ontology enrichment and pathway analysis revealed that immune-related genes dominated the overall transcriptomic signature and that metabolic processes, such as steroid biosynthesis, and metabolism of lipid and carboxylic acid, were downregulated in infected tissues. Co-expression network analysis identified transcriptional modules associated with host immune response to infection. These findings not only show how *T. gondii* infection alters porcine transcriptome in a tissue-specific manner, but also offer a gateway for testing new hypotheses regarding human response to *T. gondii* infection.

Keywords: *Toxoplasma gondii*, pig, transcriptome, host-parasite interaction, metabolism

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite, which infects nearly one-third of the world human population and all warm-blooded vertebrates (1, 2). There are three infectious stages of this parasite: tachyzoites, bradyzoites-containing tissue cysts, and sporozoites-containing oocysts (the product of sexual reproduction in the intestine of the feline definitive host). Humans acquire infection through ingestion of raw or undercooked meat, such as pork or lamb containing cysts (3, 4). Also, infection can be acquired by ingestion of food contaminated with oocysts or by exposure to soil containing oocysts (5). Despite significant research progress, our understanding of immune-related genes which are substantially involved in the pathogenesis of human toxoplasmosis remains limited. A considerable mass of research has been performed using cell culture models, which improved understanding of the pathogenesis of *T. gondii* infection. However, *in vitro* models cannot fully recapitulate *in vivo* processes.

Animal models can reduce the deficiencies that are inherent to *in vitro* models. Mice (*Mus musculus*) are the most widely model used to study *T. gondii* pathophysiology due to low cost and the availability of specific reagents (6–8). However, mice are less suitable as a model for understanding the transcriptional landscape of other mammalian species that have different transcriptional and genetic backgrounds, such as pig and humans (9). In contrast, transcriptomics and genetic technologies have shown pigs to be genetically and mechanistically relevant to study human conditions (3, 10, 11). Importantly, the common attributes of *T. gondii* infection, such as severity of infection, transplacental transmission, and interferon-gamma-related antiparasitic effector mechanisms are more similar in pigs and humans compared to the same aspects of disease in mice (4, 12–18). These facts suggest that domestic pigs (*Sus scrofa domesticus*) are more relevant model to the study of the pathophysiology of human toxoplasmosis.

Previous studies have provided beneficial but limited insights into the porcine response to *T. gondii* through using methods such as high-throughput sequencing to identify the mRNA and miRNA profiles (19–22), and microarrays (23). Recent research showed that *T. gondii* loads vary across different porcine tissues, with high parasite loads detected in the heart and lungs during acute infection, and in the heart and brain during chronic infection, regardless of the strain of the parasite (24). Therefore, genome-wide comprehensive analysis of the differential responses of pig tissues to *T. gondii* infection is required to elucidate why some porcine tissues vary greatly in their response to *T. gondii* infection.

In this study, the transcriptomic response of five different porcine tissues [brain, lung, liver, spleen, and mesenteric lymph nodes (MLNs)] to experimental *T. gondii* infection was examined using RNA-sequencing (RNA-seq). Our analysis revealed the global transcriptomic changes in relation to *T. gondii* infection at 6 and 18 days after infection. We identified hundreds of differentially expressed transcripts (DETs), infection-specific expression patterns and porcine genes that correlated with *T. gondii* load in infected pig tissues.

MATERIALS AND METHODS

Ethics and Biosafety Statement

The study design was reviewed and approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences (CAAS). The procedures involving animals were carried out in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. Animals were monitored every day for the development of clinical signs of toxoplasmosis. All efforts were made to minimize suffering and to reduce the number of pigs used in the experiment. The potentially infectious clinical and laboratory waste, such as the remaining pig tissues and *T. gondii* oocysts, were decontaminated by autoclaving prior to disposal in accordance with the local institutional health and biosafety policy on the disposal of hazardous waste.

Animals and Parasite Challenge

Twenty-four, 14-week-old, specific-pathogen-free (SPF), outbred female white pigs were purchased from Beijing Center for SPF Swine Breeding and Management. To confirm the *T. gondii*-free status of pigs before being used in the experiment, pig serum was tested using modified agglutination test (MAT) as described previously (25). The 24 *T. gondii*-seronegative pigs were randomly assigned to eight groups (3 pigs/group), which were housed in separate units. The experimental groups included two control groups (6C_1 and 6C_2) at 6 days post infection (dpi), two control groups at 18 dpi (18C_1 and 18C_2), two infected groups at 6 dpi (6I_1 and 6I_2), and two infected groups at 18 dpi (18I_1 and 18I_2). In the infected groups, each pig was infected orally with 1,000 oocysts of *T. gondii* PYS strain (genotype ToxoDB#9) in 5 ml sterile Phosphate Buffered Saline (PBS, pH 7.4). Pigs in the uninfected (control) group received 5 ml sterile PBS without any oocysts. Pigs from infected and control groups were euthanized at day 6 and day 18 post infection. These two time points post infection were chosen because pig requires 6 days to develop IgM antibodies (indicative of acute infection), whereas IgG antibodies which mark chronic infection develop after 18 dpi (26). Tissue samples were collected from cerebral cortex (thereafter called brain), liver, spleen, lung and MLNs, and were stored separately at −80°C. A total of 200 mg collected from several sites of each organ were used for RNA extraction. Confirmation of *T. gondii* infection in all collected tissues was performed by PCR, as described previously (27).

Determination of Normalized Parasite Load in Pig Tissues Using Quantitative PCR (qPCR)

DNA of pig tissues was extracted using a TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's recommendations. *T. gondii* B1 gene was used to determine normalized parasite load in pig tissues, and porcine gene coding for 18S rRNA was used to normalize the *T. gondii* B1 DNA to host DNA. Oligonucleotides for amplification of the porcine housekeeping gene 18S rRNA were: 18S-pigF (GCCTGCTGCCTTCCTTG) and 18S-pigR (ATGGTAGTCGCCGTGCC), with an expected product size of ∼109 bp. The primers used for detection of *T. gondii* B1 were: B1F (TGCATAGGTTGCAGTCACTG) and B1R (TCTTTAAAGCGTTCGTGGTC) with an expected product size of ∼131 bp. The samples with an exponential-amplification curve crossing the threshold were deemed positive for *T. gondii*, whereas samples with no amplification curve for *T. gondii* B1, but amplification of the 18S rRNA gene were considered negative for *T. gondii*. The $2^{-\Delta\Delta CT}$ method [the method can also be displayed as $2^{-(\text{CT value of target gene in tested group} - \text{CT value of housekeeping gene in tested group})}/2^{-(\text{CT value of target gene in control group} - \text{CT value of house keeping gene in control group})}$] is generally used for calculation of the fold-change of the target genes in infected relative to control samples (28). However, because *T. gondii* gene cannot be detected in the tissue that free of *T. gondii*, we used the

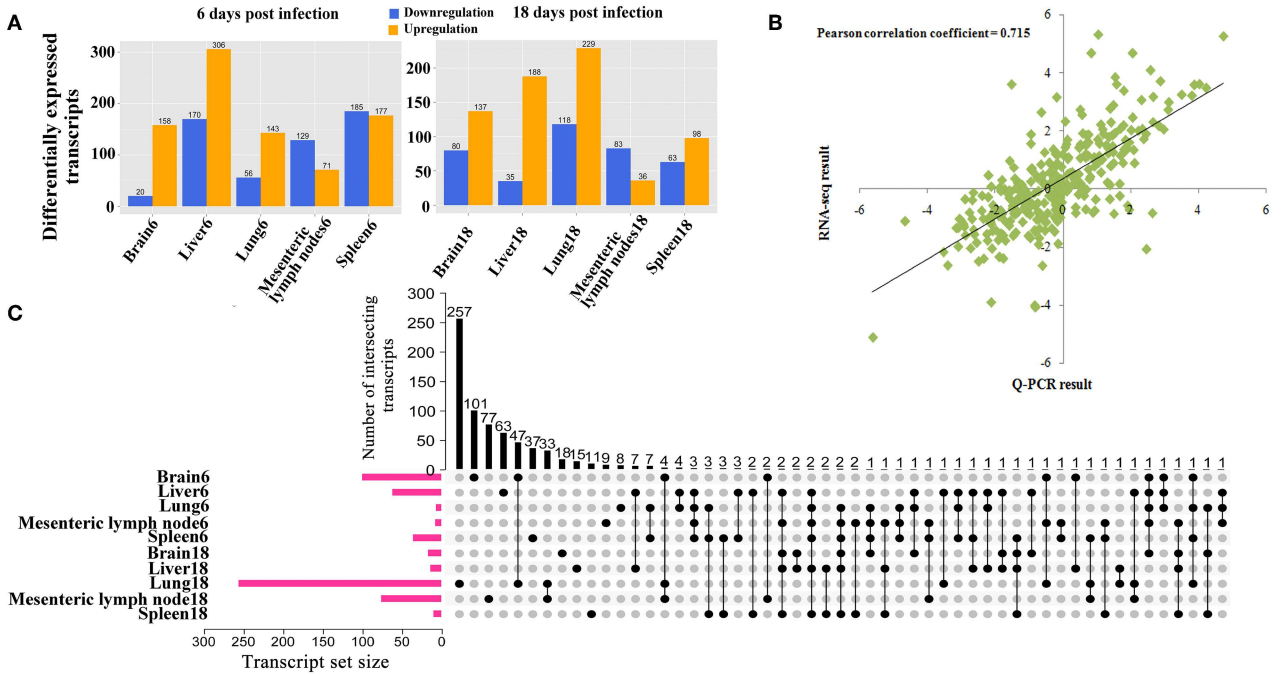

FIGURE 1 | (A) The number of differentially expressed transcripts (DETs) across different infected porcine tissues at 6 and 18 days postinfection. **(B)** Validation of RNA-seq results by qRT-PCR. Plot of gene expression (fold change) determined by the RNA-seq (X-axis) and qRT-PCR (Y-axis) of 32 selected genes (Pearson's correlation, $R^2 = 0.715$, $P < 0.01$). The fold-change of expression was expressed as log2 values. **(C)** UpSet plot showing the sets of differentially expressed transcripts from 10 different tissue samples, including the quantitative analysis of aggregate intersections between tissues. The vertical bars show the number of intersecting transcripts between tissues, denoted by the connected black circles below the histogram. The horizontal bars show the transcript set size.

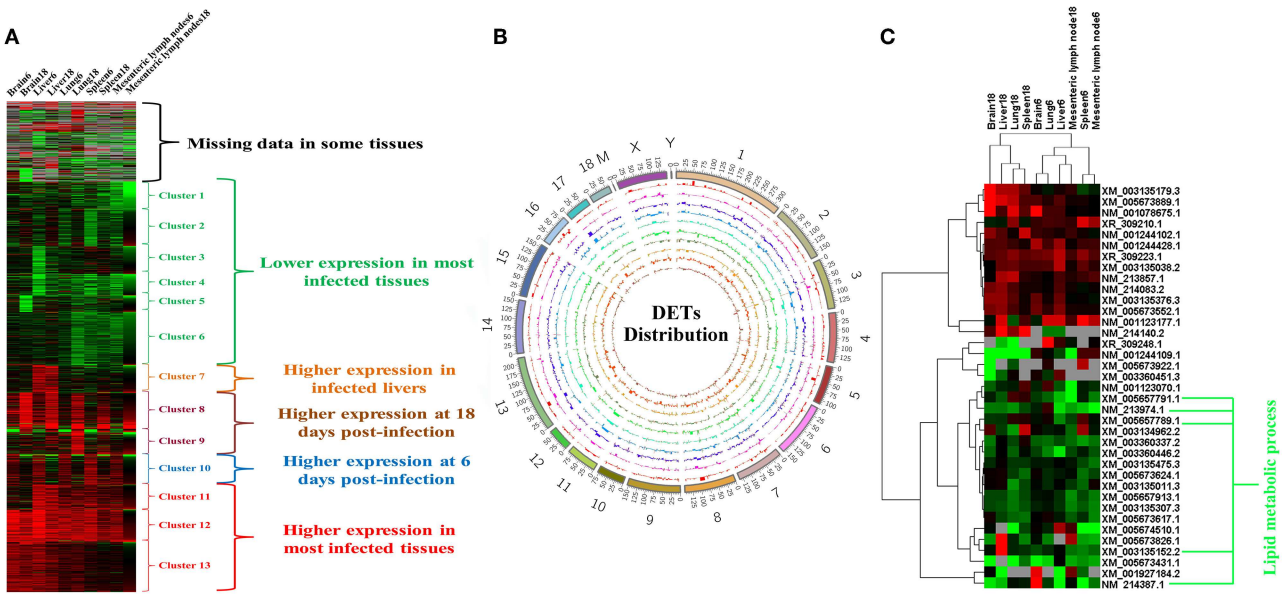

FIGURE 2 | Expression patterns of differentially expressed transcripts (DETs). **(A)** Cluster analysis of all DETs based on the average values of expression of two replicates. **(B)** The distribution of DETs across the chromosomes. From outer to inner circle represent chromosomes, DET bar plots of brain6, brain18, liver6, liver18, lung6, lung18, mesenteric lymph nodes6, mesenteric lymph nodes18, spleen6, and spleen18, respectively. **(C)** Clustering of DETs encoded by X chromosome. Red, green, and gray colors represent upregulated, downregulated and missing (undetected) data, respectively.

cycle threshold (CT) value of target and housekeeping gene to calculate the relative abundance of *T. gondii* B1 gene normalized to pig 18S rRNA gene in each infected tissue using the equation $2^{-\Delta CT}$. $-\Delta CT = -$ (CT for the targeted *T. gondii* B1 gene – CT for the pig 18S rRNA gene). qPCR was performed in a Rotor-Gene Q system (QIAGEN, Hilden, Germany) using GoTap® qPCR Master Mix (Promega, Madison, WI, USA). The cycling conditions were 95°C for 5 min followed by 50 cycles of 95°C for 10 s, 60°C for 10 s, 72°C for 15 s; the temperatures of the melt curve analysis ranged from 72 to 95°C to ensure the specificity of the amplification products.

RNA Extraction and Transcriptome Sequencing (RNA-Seq) Analysis

Total RNA of each collected tissue sample was extracted separately using TRIzol Reagent (Invitrogen China Ltd, Beijing, China) according to the manufacturer's instructions. The residual genomic DNA in the isolated RNA was removed using 20 units of RNase–Free DNase (Ambion, Shanghai, China). The integrity and quantity of RNA samples were tested with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Thermo Scientific™ Nanodrop 2000 (Wilmington, DE, USA), respectively. The RNA-seq analysis was based on two biological replicates per experimental group, and each biological replicate involved pooled RNA samples from three different pigs within each group. Although sample pooling design masks the individual response of pigs, it has been considered as a cost-efficient approach (29–32). Approximately one microgram of total RNA per each pooled sample was used as an input for the construction of mRNA library using IlluminaTruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). The transcriptome libraries were sequenced using IlluminaHiSeq™2000 according to the manufacturer's instructions. Adaptors and low quality sequencing reads were filtered using a quality cutoff score of Q20, which is widely used for quality control analysis (33–37). The resulting clean reads were mapped against the pig (*Sus scrofa domesticus*) reference genome (Sscrofa10.2) using SOAP aligner/SOAP2 software and genomic annotation data file (ref_Sscrofa10.2_top_level.gff3). The level of expression was calculated in units of reads per kilobase per million mapped reads (RPKM) (38). Expression analysis was performed using NOIseq R package (31). Transcripts with $|\log2 \text{ fold-change (FC)}| \geq 1$ and significant value >0.8 were considered differentially expressed, as per the recommendations of the NOIseq. RNA isolation, library construction, RNA sequencing, and computational analysis were performed by BGI-Shenzhen, China.

Validation of RNA-Seq Data

Thirty-two genes identified by RNA-seq analysis, across all experimental groups, were selected for validation by quantitative reverse transcriptase PCR (qRT-PCR). RNA preparations were those used for RNA-seq experiments at the corresponding time points. cDNA was synthesized from total RNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's instructions. All qRT-PCR experiments were performed in triplicate, with the housekeeping gene *GAPDH* as a control. The qRT-PCR oligonucleotide primers used in this study are described in **Supplementary Table 1**. qRT-PCR was performed in Rotor-Gene Q system (QIAGEN, Hilden, Germany) and using GoTap® qPCR Master Mix (Promega, Madison, WI, USA). qRT-PCR cycling conditions were as follows: 95°C for 5 min followed by 50 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s; the temperatures of the melt curve analysis ranged from 72 to 95°C to ensure the specificity of qRT-PCR products. The $2^{-\Delta\Delta CT}$ relative expression method was used to calculate gene expression.

Gene Ontology (GO) Enrichment and KEGG Analysis

GOseq package (v1.22) in R (www.r-project.org) was used for Gene Ontology (GO) enrichment analysis, such as biological process (BP), cell component (CC), molecular function (MF). Pathway analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Significantly enriched GO terms or pathways were identified using hypergeometric test followed by FDR correction method (39). The FDR corrected $P < 0.05$ was used as cutoff for the significantly enrichment GO terms or pathways. All differentially expressed transcripts (DETs) were clustered with log2 fold-change of DETs using Gene Cluster 3.0 and Euclidean distance. We used Upset (40) to visualize intersecting sets in order to identify the unique and common DETs across 10 tissue subsets.

Coexpression Network and Correlation Analysis

We performed coexpression analysis, which has been widely applied to identify genes involved in host-parasite interaction (41–43). The weighted gene correlation network analysis (WGCNA) *R* package (44) was used to establish a correlation matrix between pig mRNA expression and normalized *T. gondii* load in each infected tissue. WGCNA was performed as the network construction and module detection protocol in the WGCNA R package (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/index.html). RPKMs of all transcripts were used as input data. We have chosen a soft-thresholding power of 14 because this was the lowest power needed to exceed a scale-free topology fit index of 0.75. Days post infection, infection status and normalized *T. gondii* load were used as input traits in the module-trait association analysis. The cluster of highly interconnected genes that share a similar expression pattern was considered as a coexpression module. Multidimensional scaling (MDS) plot was constructed to visualize pairwise relationships specified by a dissimilarity matrix, indicating dissimilarity/similarity based on gene expression data. The hub gene of coexpression module was identified based on a high module membership value or connectivity (i.e., the sum of connection strengths with the other module genes). Generally, the hub genes of specific module are located at the finger tip of MDS plot. For further testing of the predictive performance between the host gene expression and *T. gondii* load, pROC package was used to perform receiver operating characteristic (ROC)

curve analysis, and to calculate the area under the ROC curve (AUC), a performance metric, which is generally used for the identification of potential biomarkers. The gene in coexpression module that was significantly correlated with *T. gondii* load, and had significant $P < 0.05$, RPKM > 1, AUC > 0.6, was considered as a host gene significantly correlated with *T. gondii* load (HGSCTG). HGSCTG genomic hotspots were defined on the basis of >5 HGSCTG per 10 Mb genomic region. Gene regulatory networks were reconstructed using the coexpression data and TRRUST database, and were visualized with Cytoscape software. The regulatory transcription factors were identified as the ones that co-express with their target genes. Finally, the relationships between enzymes and substrates were analyzed using the online DrugBank database (https://www.drugbank.ca/).

Accession Numbers

The RNA-Seq datasets described in this study have been deposited in NCBI Short Read Archive database (https://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR6203124 to SRR6203163.

RESULTS

Confirmation of *T. gondii* Infection and Normalized Parasite Load in Pig Tissues

At 6 dpi, all pigs in the infected groups exhibited clinical signs, such as fever and inappetence, whereas pigs in the control groups remained clinically healthy. The brain, liver, spleen, lung, and MLNs of infected pigs were all PCR-positive, whereas no *T. gondii B1* gene amplification was achieved in corresponding tissues from uninfected (control) pigs. Infected lungs showed the highest *T. gondii* load (**Supplementary Table 2**).

Transcriptomic Features and Validation of RNA-Seq Results

The RNA integrity numbers (RINs) of all RNA templates were >8.0. Also, 99% of the reads showed high quality values > Q20, and 90% of the clean reads were up to Q30 (**Supplementary Figures 1–3**). More than 62 million clean reads were obtained from each tissue sample and more than 32,000 transcripts were detected (**Supplementary Table 3**). At 6 dpi, 178, 476, 199, 200, and 362 DETs were detected in infected

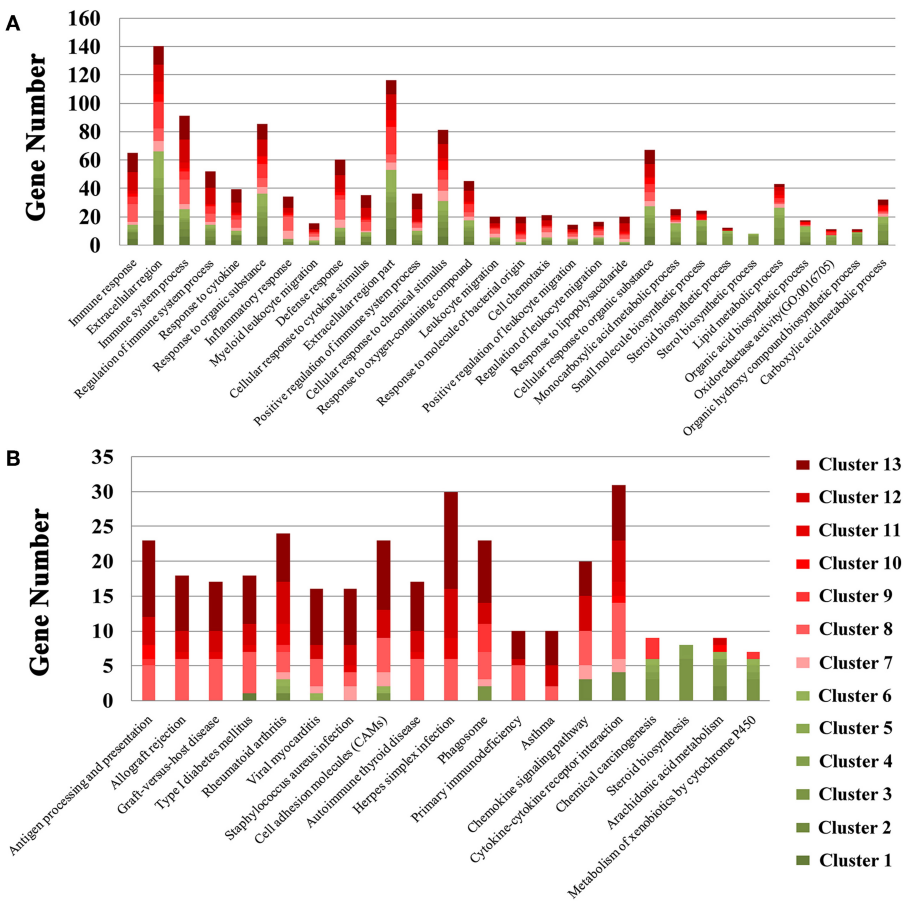

FIGURE 3 | Stacked bar plots showing the results of GO enrichment **(A)** and KEGG analysis **(B)** of genes in the 13 clusters identified in **Figure 2A**.

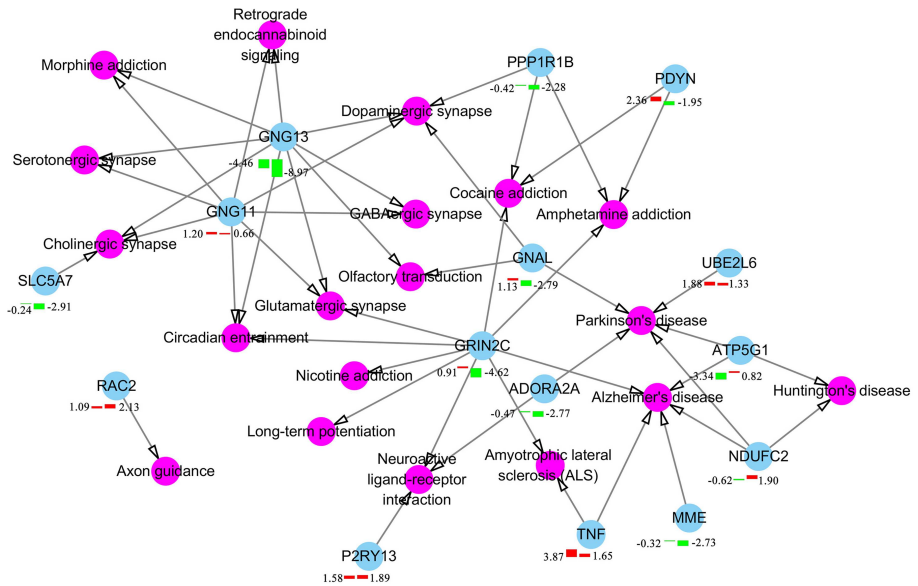

FIGURE 4 | Differentially expressed components of neuron signaling pathways in *T. gondii* infected brain. Pink color represents neuron signaling pathway and cyan color represents the differentially expressed transcripts in brain at 6 or 18 dpi. The bars underneath the nodes represent the change in the expression level (left and right bars represent expressional changes at 6 and 18 dpi, respectively). Upregulated and downregulated transcripts are represented by red and green colors, respectively. The log2 fold-change of transcripts are described together with the bars.

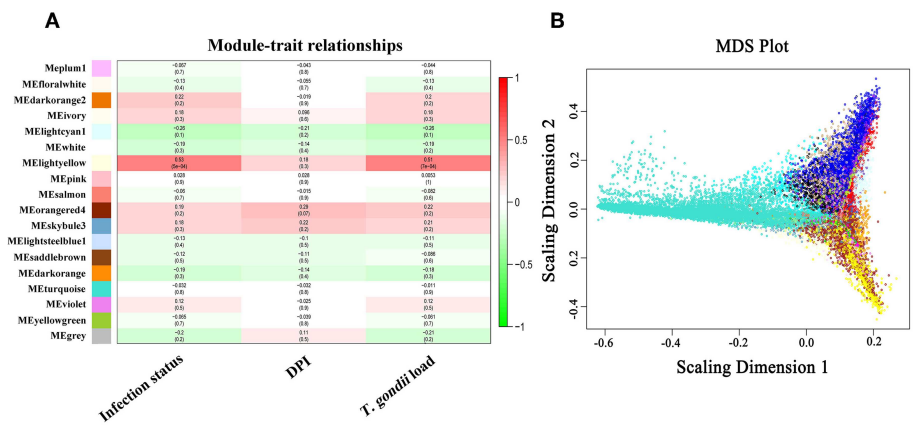

FIGURE 5 | (A) Module and traits relationships. The color-coded legend shows the correlation degree, with positive and negative numbers indicating positive and negative relationship, respectively **(B)**. The classical multidimensional scaling plot of all transcripts.

brain, liver, lung, MLNs and spleen, respectively; whereas at 18 dpi, 217, 223, 347, 119, and 161 DETs were found in the infected brain, liver, lung, MLNs and spleen, respectively (**Figure 1A**). The global Pearson correlation coefficient between qRT-PCR results and RNA-seq results was 0.715 (**Figure 1B**), suggesting a good agreement between results obtained by the two methods, supporting the validity of the transcriptomic RNA-seq data. After intersecting the differentially expressed transcript sets across tissues, there was not any DET shared across all tissues as shown in the vertical bars and the connected black circles below the histogram (**Figure 1C**), suggesting a lack of commonly DETs shared across the analyzed tissues. According to Euclidean distance, as shown in **Figure 2A**, all DETs were clustered into five distinct expression patterns, including (pattern 1) low expression in most infected tissues (downregulated in ≥ 6 tissue samples at 6 and 18 dpi), (pattern 2) high expression in infected liver only, (pattern 3) high expression at 18 dpi, (pattern 4) high expression at 6 dpi, and (pattern 5) high expression in most infected tissues (upregulated in ≥ 6 tissue samples at 6 and 18 dpi). The distribution of DETs across chromosomes is shown in **Figure 2B**. Three chromosomes encoded most of the DETs: chromosome 2 (110 DETs), chromosome 6 (108 DETs), and chromosome 7 (114 DETs). No DETs were found in the mitochondrial DNA or chromosome Y. However, 37 DETs

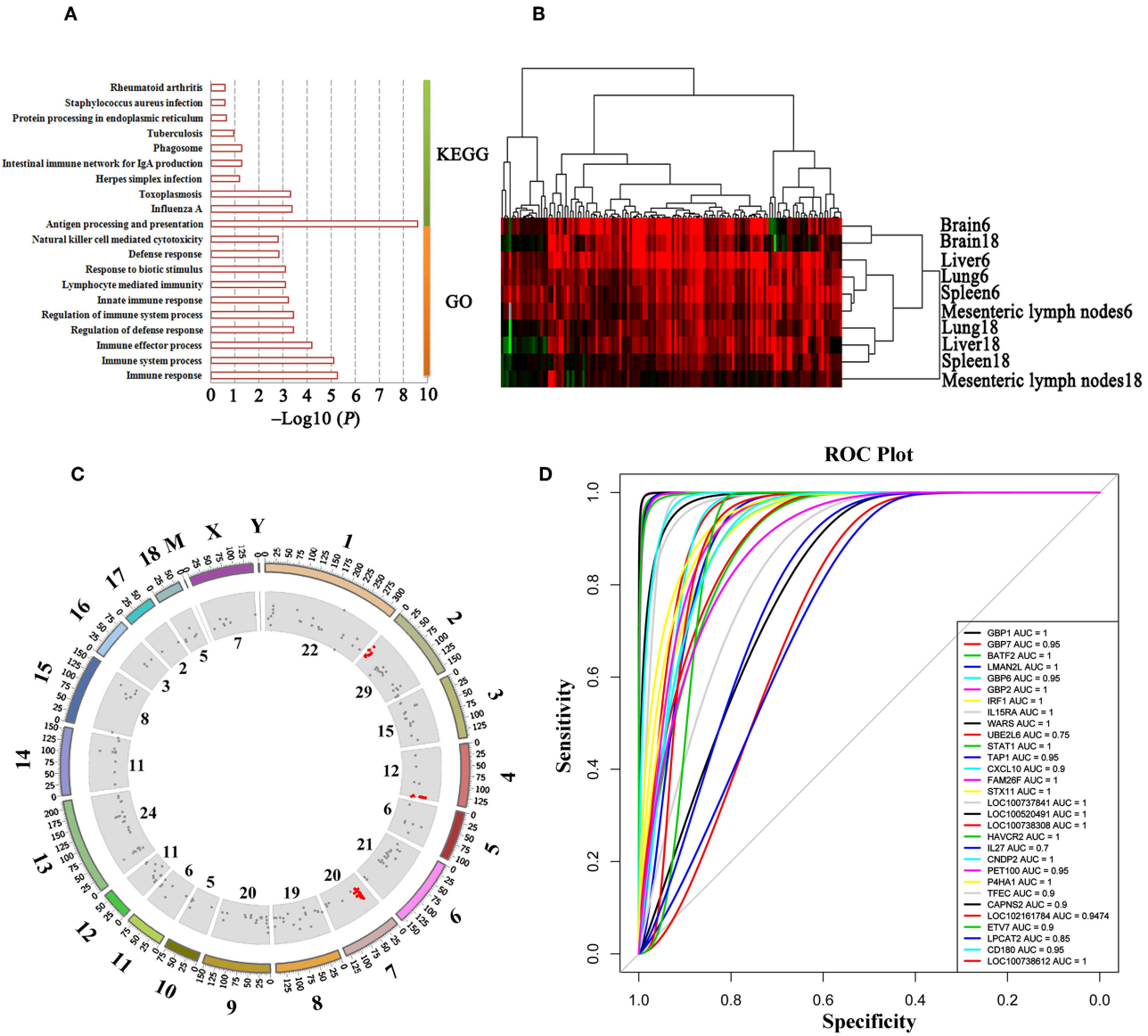

FIGURE 6 | Functional annotation and transcript expressional cluster of MElightyellow module. **(A)** GO and KEGG enrichment of transcripts in MElightyellow module. The top 10 GO terms are listed according to the enrichment *P*-value. **(B)** Expressional clustering of transcripts in MElightyellow module. Upregulated, downregulated, and missing transcripts are denoted by red, green, and gray colors, respectively. **(C)** Distribution of the host genes significantly correlated with *T. gondii* load (HGSCTG) on the pig genome. Outer circle represents the chromosome, scatter in inner circle represents the distribution of HGSCTG, the numbers adjacent to inner circle represent the number of HGSCTG that are encoded by the chromosome, red scatter shows HGSCTG distributed on the hotspot sites. **(D)** ROC tests between the top 30 hub gene expressions and *T. gondii* parasite DNA load.

were encoded by chromosome X and were clustered into two expression patterns (**Figure 2C**).

GO Enrichment and KEGG Analysis

We analyzed the functional enrichment and significant pathways associated with the DETs clustered in the five expression patterns. The downregulated transcript cluster in most infected tissues (pattern 1) was significantly enriched for GO terms involved in metabolic or tissue development processes, such as proteinaceous extracellular matrix, lipid metabolic process, animal organ development, PPAR signaling pathway, and metabolism of xenobiotics by cytochrome P450 (**Supplementary Table 4**). In the upregulated transcript clusters in infected liver (pattern 2) and most infected tissues (pattern 5), most of the enriched transcripts were related to GO terms or pathways involved in immune response. These included cytokine receptor binding, regulation of interleukin (IL)-12 production, Jak-STAT signaling pathway, nuclear factor kappa B (NF-κB) signaling pathway,

TABLE 1 | The genomic location of the host genes significantly correlated with *T. gondii* load (HGSCTG) in the HGSCTG hotspots.

Gene symbol	Chromosome	Start site	End site	Mean RPKM	AUC
IRF7	Chromosome 2	299457	302151	4.7	0.65
LOC102161205	Chromosome 2	2828704	2838699	2.99	0.95
TCIRG1	Chromosome 2	3476372	3488276	1.2	0.65
LOC100736864	Chromosome 2	6230389	6231678	1	1
BATF2	Chromosome 2	6312368	6321030	1.85	1
STIP1	Chromosome 2	6978503	7040367	194	0.7
FAM111A	Chromosome 2	11864850	11873989	1.3	0.8
UBE2L6	Chromosome 2	12987538	13002246	143.7	0.75
LOC102161784	Chromosome 4	139395369	139411664	2.46	0.95
LOC100737841	Chromosome 4	139499543	139509159	2.77	1
LOC100523668	Chromosome 4	139559304	139570934	2.45	1
GBP7	Chromosome 4	139582087	139601514	123.4	0.95
GBP2	Chromosome 4	139612348	139676554	14.9	1
GBP1	Chromosome 4	139651545	139667272	179.1	1
LOC100622791	Chromosome 7	24603720	24604965	2751.5	1
SLA-3	Chromosome 7	24641613	24645323	772.3	1
UBD	Chromosome 7	25330502	25332256	118.5	1
TAP2	Chromosome 7	29412165	29423473	18.1	0.95
TAP1	Chromosome 7	29429379	29438656	5.35	0.95
PSMB9	Chromosome 7	29438820	29443905	46	1
SLA-DMB	Chromosome 7	29485890	29491702	104.3	1
SLA-DMA	Chromosome 7	29500107	29504539	42.7	0.9
ETV7	Chromosome 7	36987742	37008910	15.6	0.9
PIM1	Chromosome 7	37691993	37697470	41.4	0.95

Analysis is based on the pig reference genome (Sscrofa10.2) and the annotation file (Sscrofa10.2_top_level.gff3).

lymphocyte chemotaxis, IL-8 secretion, and cytokine secretion (**Supplementary Tables 5**, **6**). The cluster containing upregulated transcripts at 6 dpi (pattern 4) was not enriched in any GO term. However, most significantly enriched GO terms in the transcript's cluster with high expression pattern at 18 dpi (pattern 3) were related to immune response, such as antigen processing and presentation pathway, lytic vacuole, response to cytokine, lymphocyte-mediated immunity, and chemokine-mediated signaling pathway (**Supplementary Table 7**). Only five transcripts encoded by X chromosome were involved in lipid metabolic processes (**Figure 2C**).

We also performed GO enrichment and KEGG analysis of all DETs. According to FDR corrected *P*-value, the top 30 significantly enriched GO terms and pathways are shown in **Figure 3**. Most of the transcripts that were significantly enriched in immune-related GO terms or pathways belonged to clusters 7–13, which had upregulated expression patterns (**Figure 2A**). However, most of the transcripts that were significantly enriched in metabolic related GO terms or pathways belonged to clusters 1–6, which had low expression patterns (**Figure 2A**). In the infected brains, 19 neural signaling pathways were regulated by 15 DETs, including *Adora2a*, *P2ry13*, *Grin2c*, *Gng11*, *Gng13*, *Ppp1r1b*, *Pdyn*, *Gnal*, *Rac2*, *Atp5g1*, *Ndufc2*, *Slc5a7*, *Ube2l6*, *Tnf*, and *Mme* (**Figure 4**).

Coexpression and Regulatory Network Analysis

The soft-threshold power, that exceeded a scale-free topology fit index of 0.75 for each network was 14 (**Supplementary Figure 4**). Therefore, number 14 was chosen as the soft power threshold for constructing WGCNA. As shown in **Figure 5A**, 18 modules, including gray module, denoting unassigned transcripts, were found. The module-trait association shows that MElightyellow was significantly correlated with infection status and *T. gondii* load (**Figure 5A**). The global correlation coefficient between MElightyellow and *T. gondii* load was 0.51 with a *P*-value of 7e-04. Details of the transcripts and module relationships are summarized in **Supplementary Table 8**. Multidimensional scaling (MDS) plot shows that the transcripts of each module were successfully categorized by coexpression analysis (**Figure 5B**). Most of the genes in MElightyellow module were upregulated in most infected tissues, and significantly enriched in immune-related biological processes (**Figures 6A,B**). The genomic locations of genes in the MElightyellow module are shown in **Figure 6C**.

We identified 152 genes (165 transcripts in total) as HGSCTG that were significantly correlated with *T. gondii* loads. The details of HGSCTG are listed in **Supplementary Table 9**. Chromosome 2 encodes most of the MElightyellow module genes (29 genes) and these genes were distributed on 3 spots, including head spot (chromosome 2: 0Mb-13Mb), which encodes 10 genes (8 of these were HGSCTG), middle spot (chromosome 2: 53–84 Mb), which encodes 10 genes (8 of these were HGSCTG), and end spot (chromosome 2: 124–162 Mb), which encodes 9 genes (4 of these were HGSCTG). Chromosome 4: 139–144 Mb encodes 6 HGSCTG and chromosome 7: 22–38 Mb encodes 10 HGSCTG. Therefore, chromosome 2: 0.3–13 Mb, chromosome 4: 139–144 Mb, and chromosome 7: 22–38 Mb were identified as HGSCTG genomic hotspots. The details of HGSCTG locations in the hotspots are shown in **Table 1**. Results of the ROC analysis of the top 30 hub genes of MElightyellow module are shown in **Figure 6D**. All the areas under ROC curve (AUC) were >0.7 and the majority of them were >0.9, indicating a high correlation between the top 30 hub gene expression and *T. gondii* parasite DNA load. Functions of the hub genes are described in **Table 2**.

Differentially Expressed Transcription Factors (TFs) and Their Regulatory Networks

We detected 31 TFs in the infected brain, liver, lung, spleen, and MLN. These 31 TFs were grouped into 2 clusters (**Figure 7A**). The upregulated TF cluster included *Fos*, *Hopx*, *Zbtb16*, *Mycl*, *Junb*, *Litaf*, *Stat3*, *Tead4*, *Foxs1*, *Batf*, *IRF8*, *BATF2*, *IRF7*, *IRF1*, *Tfec,* and *Stat1*. The downregulated TF cluster included *Msc*, *Id4*, *Dlx5*, *Dlx1*, *Dlx2*, *Pbx3*, *Tcf21*, *Mycn*, *Sox17*, *Smad6*, *Hes1*, *Etv5*, *Gli1*, *Barx1,* and *Nr0b1*. We found the target genes to 18 TFs in the TRRUST database, including *Stat3*, *Stat1*, *Fos*, *Irf1*, *Mycn*, *Gli1*, *Junb*, *Msc*, *Irf8*, *Hes1*, *Zbtb16*, *Irf7*, *Nr0b1*, *Dlx5*, *Id4*, *Hopx*, *Tcf21,* and *Tead4*. According to TRRUST database, 240 DETs were regulated by 15 differentially expressed TFs (**Figure 7B**). As shown in **Figures 7C–F**, Irf1 was co-expressed with and may

function as a regulator for seven target genes (*Cxcl10*, *Ciita*, *Il27*, *Psmb9*, *Socs1*, *Tap1*, and *Tap2*); Stat1 was co-expressed with 12 target genes (Cxcl10, Ciita, Il27, Psmb9, Socs1, Tap1, Hsp90aa1, Jak2, Pim1, Tnfsf13b, Irf7, and Irf1); Irf8 was co-expressed with *Ncf2*; and Stat3 was co-expressed with *Il2ra*, *Mcl1*, *Pias3*, and *Usp7*.

Cytokine Expression

We detected 38 cytokines and 21 cytokine receptor-related transcripts that were differentially expressed, including 18 differentially expressed chemokines and seven differentially expressed chemokine receptors. Most of these were upregulated in the infected tissues (**Figure 8A**), including four HGSCTG (*Cxcl10*, *Il27*, *Il15*, and *Il15ra*). In infected tissues, upregulation of chemokines and chemokine receptors increases the chemotaxis of 20 immune cells, such as DC, NK, macrophage, and T cells (**Figure 8B**).

Comparative Toxicogenomic Analysis

We found that 45 DETs were involved in xenobiotics or drug metabolism (**Figure 8C**). Most of these were downregulated, especially in the liver at 6 dpi. We also found that the DETs encode enzymes that metabolize 330 substances or drugs, such as ethanol, acetaminophen, ketoconazole, phenobarbital, and benzyl alcohol. The relationship among 330 xenobiotic substances and DETs related to metabolism are shown (**Supplementary Table 10**).

DISCUSSION

We compared the transcriptomes of *T. gondii*-infected and uninfected pigs using RNA-seq approach. Hundreds of transcripts were differentially expressed in the porcine brain, liver, lung, spleen, and MLNs at 6 and 18 dpi (**Figure 1**). These DETs were distributed on all the pig chromosomes, but not the mitochondrial genome and chromosome Y (**Figure 2B**).

We tested whether transcriptomic changes overlap across porcine tissues. As shown in **Figure 1C**, there was no common DET in the 10 tissue groups. Next, we characterized the transcriptomic changes in infected tissues using gene clustering analysis, which showed that all DETs are clustered into five different expressional patterns (**Figure 2A**). Functional enrichment analysis of the downregulated transcripts in most infected tissues revealed that downregulation of metabolism-related and tissue development-related transcripts is prominent in infected tissues (**Supplementary Table 4** and **Figure 3**). This finding may have clinical relevance. During pregnancy, mother-to-fetus transmission of *T. gondii* can occur, resulting in abortion, stillbirth, or congenital malformation (71). It remains to be determined if the downregulation of these transcripts observed in pigs can also occur in the fetus if they become congenitally infected. Mindful of the fact that successful pregnancy requires delicate immune balance to protect the fetus, the deleterious effects of *T. gondii* induced-inflammatory response mediated by increased expression of cytokines and cytokine receptor-related transcripts (**Figure 8A** and **Supplementary Table 6**) may cause undesirable health consequences in the fetus.

Forty-three genes involved in lipid metabolic processes, including 26 genes belonging to clusters 1–6 (**Figure 3A**) showed lower expression in infected tissues (**Figure 2A**). Five of these were encoded by chromosome X (**Figure 2C**). These results suggest that, during *T. gondii* infection, chromosome X contributes ∼ one-eighth of the downregulated genes involved in lipid metabolism. The downregulation of metabolic terms or pathways is consistent with our previous proteomic and transcriptomic investigations in mice (72–74), suggesting that downregulation of metabolic processes may also occur in other mammalian hosts.

T. gondii influences mouse behavior via altering glutamate transporter Slc1a2 (also known as GLT-1) (75) and GABA signaling pathway (76). In our study, *Slc1a2* was not differentially expressed in the brain. However, three genes (*Gng11*, *Gng13,* and *Grin2c*) involved in the signaling mechanism of glutamatergic and GABAergic synapse were differentially expressed. This indicates that *T. gondii* may alter pig behavior by interfering with glutamatergic and GABAergic synapse pathways via altering the expression of *Gng11*, *Gng13,* and *Grin2c*. We also found 19 neural synapse signaling pathways altered in infected brains (**Figure 4**), such as olfactory transduction pathway, which may alter the sense of smell of infected pigs. Chronically infected rodents exhibit behavioral changes, such as loss of aversion and even attraction to cat odors (77). In humans, infection with *T. gondii* has been associated with behavioral abnormalities (78) and increased risk of developing psychiatric disorders (79).

We further investigated which host genes are significantly correlated with *T. gondii* load (HGSCTG) using WGCNA analysis, which identified 18 coexpression modules (**Figure 5** and **Supplementary Table 8**). By relating modules to sample traits (days after infection, infection status, and *T. gondii* load in tissues), MElightyellow module was significantly correlated with parasite load in infected tissues (**Figure 5A**). Most of the transcripts in MElightyellow module were upregulated in infected tissues (**Figure 6B**). As shown in **Figure 6A**, genes in MElightyellow module were significantly enriched in immune response and infection-related terms or pathways. By combining WGCNA coexpression and ROC curve analyses, we identified 152 HGSCTG (**Supplementary Table 9**), including three HGSCTG cytokines (*Cxcl10*, *Il27*, *Il15*) and one cytokine receptor (*Il15ra*). These four genes seem to be important for mice survival during *T. gondii* infection (**Table 2**).

Three HGSCTG genomic hotspots (**Figure 6C**) encoding 24 HGSCTG (**Table 1**) were also identified. Most of these 24 genes were involved in immune processes, and some have anti-*T. gondii* activity, such as *Gbp1*, *Gbp2*, *Gbp7*, *Batf2*, and *Tap1*. As shown in **Figure 6D**, the AUC of the top 30 hub genes in the MElightyellow module was >0.7 and for most of these genes, the AUC was >0.9, indicating a strong correlation between the top 30 hub gene expression and *T. gondii* DNA load. This result shows a synergy between the results obtained by ROC and WGCNA analysis, suggesting that the identified hub genes (*CD180*, *STX11*, *FAM26F*, *TFEC*, *ETV7*, *LOC100738612*, and *LOC100520491*) are HGSCTG.

We detected 31 differentially expressed TFs, grouped into upregulated and downregulated clusters (**Figure 7A**). In these

TABLE 2 | Description of mouse or human orthologs of the top 30 hub genes of MElightyellow module.

Gene symbol	Mean RPKM	q. weighted	cor. weighted	Gene function	References
STAT1*	400.5	1.93E-10	0.88	Mediation of the production of MHC, NO, and IFN-inducible GTPase family of proteins that function as anti-*T. gondii* factors directly	m (45)
IRF1*	234.47	9.36E-11	0.88	Cis-acting factor of iNOS which is needed for *T. gondii* control	m (46)
BATF2*	1.85	2.3E-11	0.9	Batf2 play regulatory role in CD8α + classical DC development and contributes to anti-*T. gondii in vivo* infection.	m (47)
CXCL10*	692.79	3.62E-10	0.87	CXCL10 is required to maintain T-cell populations and to control parasite replication during chronic ocular Toxoplasmosis	m (48)
GBP2*	14.9	4.11E-11	0.89	Accumulate around the PV of *T. gondii* and contributes to anti-*T. gondii* in mice.	m (49)
GBP1*	179.1	1.31E-11	0.91	Accumulate around the PV of *T. gondii* and contributes to anti-*T. gondii* in mice.	m (50)
GBP6*	21.6	4.11E-11	0.89	GBP6 accumulate around the PV of *T. gondii* and contributes to anti-*T. gondii.*	m (50)
GBP7*	123.4	2.3E-11	0.90	GBP7 accumulate around the PV of *T. gondii* and contributes to anti-*T. gondii.*	m (50)
IL15RA*	10.6	1.14E-10	0.88	IL15 signal pathway contributes to the development of antigen-specific memory CD8+ T cells against *T. gondii*.	m (51)
TAP1*	5.35	2.11E-10	0.88	TAP1 is one subunit of the transporter associated with antigen processing. TAP-1 indirectly regulates CD4+ T cell priming in *Toxoplasma gondii* infection by controlling NK cell IFN-γ production.	m (52)
IL27*	8.16	1.88E-9	0.86	Interleukin 27 regulates Treg cell populations that required to limit *T. gondii* infection-induced pathology.	m (53, 54)
UBE2L6*	143.7	1.44E-10	0.88	Ubiquitination targets *T. gondii* for endo-lysosomal destruction.	h (55)
WARS	2.3	1.34E-10	0.88	WARS is one component of primary defense system against infection.	m (56)
CD180	10.95	6.08E-9	0.84	CD180/MD1 complex is a member of the Toll-like receptor (TLR) family and it plays a crucial role in the response of immune cells to LPS.	h (57)
LMAN2L	29.77	2.63E-11	0.9	Export of glycoproteins	h (58)
HAVCR2	2.5	1.88E-9	0.86	Positive regulation of tumor necrosis factor secretion	h (59)
LPCAT2	4	5.63E-9	0.85	LPCAT2 is a critically important enzyme for the biogenesis of proinflammatory lipid mediator (Platelet-activating factor) and the membrane homeostasis of inflammatory cells. It can also catalyze the formation of phosphatidylcholine.	h (60, 61)
CNDP2	4	1.98E-9	0.86	CNDP2 is a cytosolic non-specific dipeptidase	h (62)
STX11	55.4	1.14E-9	0.86	In human, STX11 is part of the cytolytic machinery of T and NK cells and involved in the fusion of lytic granules with the plasmamembrane.	h (63)
FAM26F	41.3	4.61E-10	0.87	FAM26F contains an immunoglobin (Ig) like fold and it is expressed on various immune cells, playing a role in infection and immunity.	h (64)
PET100	17	2.05E-9	0.85	PET100 is a molecular chaperone required for the assembly of cytochrome c oxidase.	h (65)
TFEC	4.6	4.49E-9	0.85	TFEC is specifically induced in bone marrow-derived macrophages upon stimulation with the Th2 cytokines.	m (66)
P4HA1	26.7	4.03E-9	0.85	P4HA1 residing within the lumen of the endoplasmic reticulum (ER) and in charge of catalyzing formation of 4-hydoxyproline in collagens. It is essential for mice survival.	m (67)
LOC100738612	21.3	7.25E-09	0.85	Also known as ATF3. Negative regulation of iNOS expression and NO production in activated macrophages to avoid pathogenesis of tissue damage.	m (68)
ETV7	15.66	5.59E-09	0.85	ETV7 is a transcription factor and involved in proliferation and survival of normal mouse B cells.	m (69)
CAPNS2	1.2	4.49E-09	0.85	CAPNS2 is one of cysteine proteases.	m (70)
LOC100737841	2.77	1.18E-09	0.86	LOC100737841 is guanylate-binding protein 2-like protein	p NCBI annotation
LOC100738308	4	1.35E-09	0.86	LOC100738308 is signal transducer and activator of transcription 1-like protein	p Pig genomic annotation file
LOC100520491	98.3	1.31E-09	0.86	According to PSI-BLAST, it homologous to rat Klrb1b which involved in anti-malaria.	p PSI-BLAST and EggNOG 4.5 annotation
LOC102161784	2.46	4.99E-09	0.85	Guanylate-binding protein.	p PSI-BLAST and EggNOG 4.5 annotation

**Indicates function has been confirmed in mice or human.*
q. Weighted: Weighted p-value corrected with FDR;
cor. Weighted: correlation of T. gondii load with gene expression weighted by a network term.
m: denotes annotation from mice.
h: denotes annotation from human.
p: denotes annotation from pig.

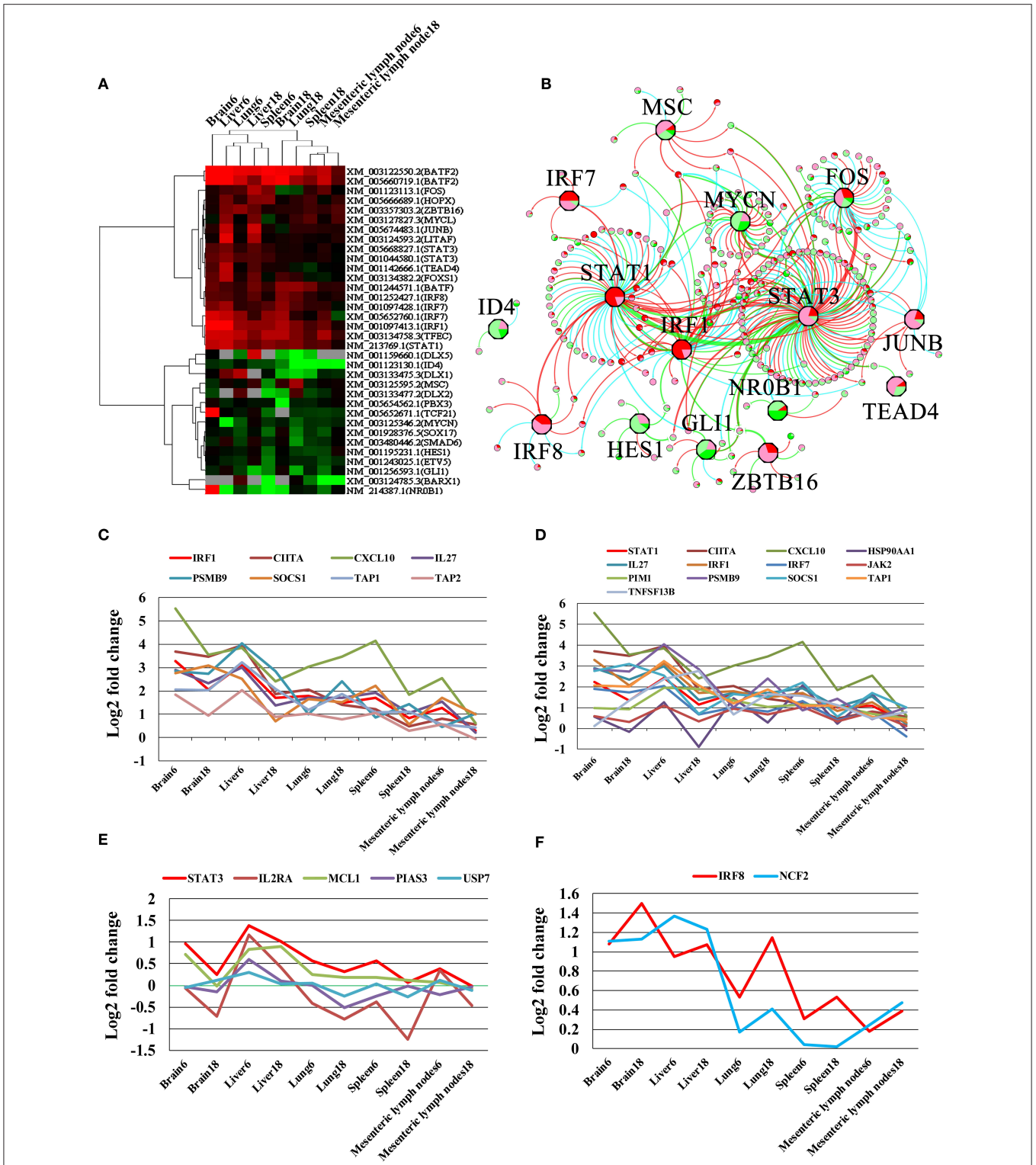

FIGURE 7 | Differentially expressed transcription factors (TFs) and their gene targets. **(A)** Cluster of all differentially expressed TFs. Upregulated, downregulated, and missing transcripts are denoted by red, green, and gray colors, respectively. **(B)** Differentially expressed TFs and their target genes. The octagon nodes represent TF and the circular nodes represent target gene of TF with the pie charts inside the nodes denoting the proportion of expressional change in infected samples. Pie chart colors represent the levels of gene expressional changes: red (log2 fold-change ≥ 1), green (log2 fold-change ≤ -1), pink (log2 fold-change between 0 and 1), light green (log2 fold-change between 0 and -1). **(C–F)** Co-expressed target genes of Irf1, Stat1, Stat3, and Irf8, respectively.

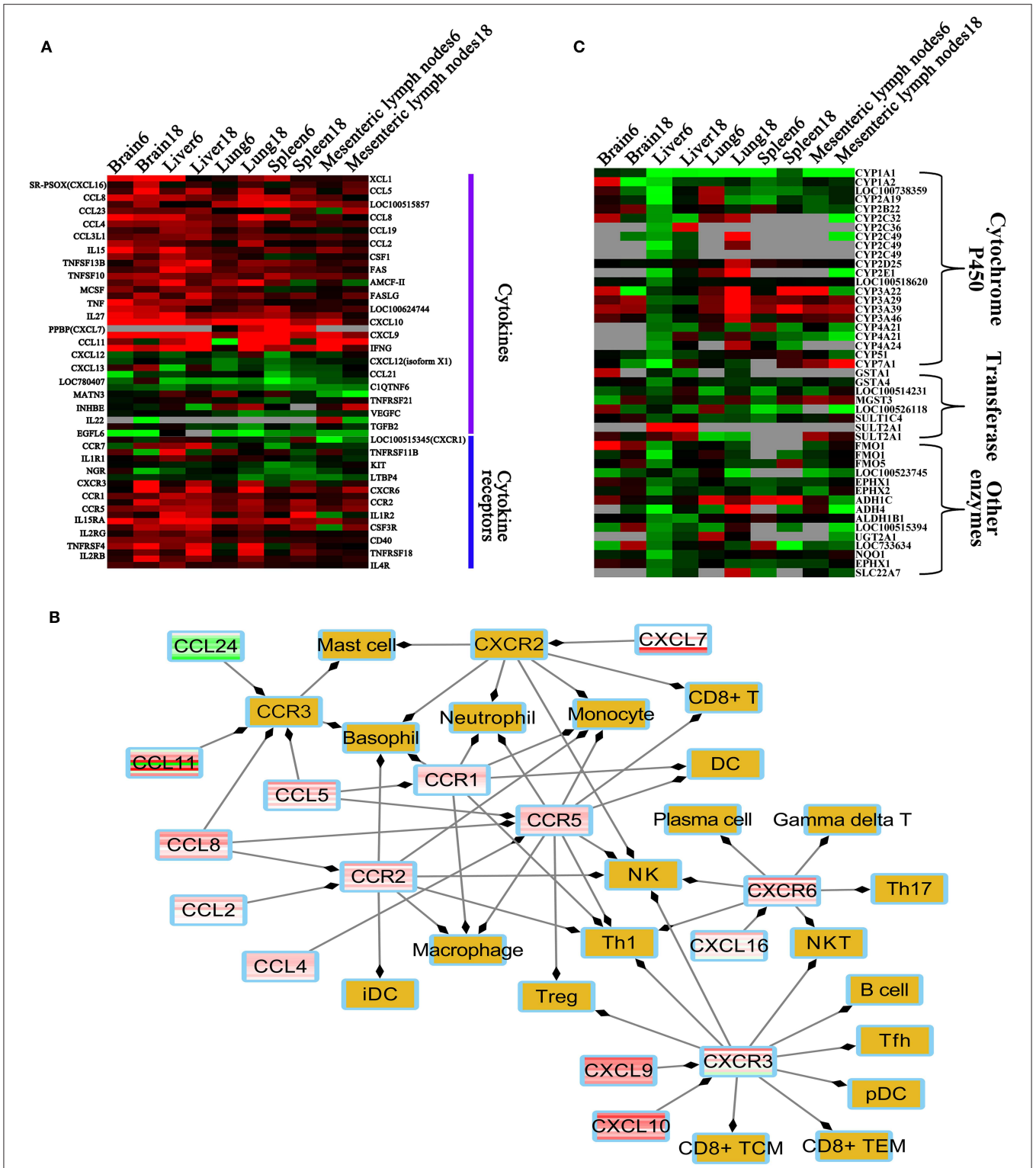

FIGURE 8 | Heatmaps of differentially expressed metabolic enzymes, cytokine and cytokine receptors. **(A)** Heatmap of the differentially expressed cytokines and cytokine receptors. **(B)** Immune cells regulated by differentially expressed cytokines or cytokine receptors. **(C)** Heatmap of differentially expressed enzymes involved in metabolism. Red, green, and gray colors represent upregulated genes, downregulated genes, missing genes, respectively. Brown color denotes non-differentially expressed chemokine receptors and immune cells. Abbreviations: Th, T helper cell; Treg, regulatory T cell; iDC, immature dendritic cell; DC, dendritic cell; pDC, plasmacytoid dendritic cell; NK, natural killer; NKT, natural killer T cell; TCM, central memory T cell; TEM, effector-memory T cell; Tfh, T follicular helper cell.

differentially expressed TFs, Batf2, Irf7, Irf1, Tfec, and Stat1 were HGSCTG. We also found hundreds of gene targets to 15 differentially expressed TFs in the TRRUST database (**Figure 7B**). The coexpression analysis revealed that 12, 7, 1, and 4 targeted genes shared similar expression pattern with Stat1, Irf1, Irf8, and Stat3, respectively (**Figure 7**). The Irf1 and Stat1 contribute to *T. gondii* control via regulating the expression of factors essential for host resistance to infection, such as TAP complex (80), Cxcl10, Ciita (81), Il27, and Jak2 (82). We also identified 38 cytokines and 21 cytokine receptor-related transcripts that were differentially expressed. Most of these were upregulated in infected tissues (**Figure 8A**). In agreement with others (83), upregulation of these genes can increase chemotaxis of 20 immune cells, including DCs, NK cells, macrophages, and T cells in most infected tissues (**Figure 8B**). These immune cells, which play important roles in *T. gondii* control (6), can be regulated by CXCl9, CXCl10, and CXCR3 signaling pathways (**Figure 8B**), contributing to the pig immune response to *T. gondii* infection.

As shown in **Figure 8C**, 45 DETs were involved in xenobiotics or drug metabolism, most of these were downregulated in infected tissues, especially in the liver at 6 dpi. According to the DrugBank database, 330 xenobiotics or drugs were found to be metabolized by enzymes coded by these DETs (**Supplementary Table 10**). Acetaminophen is used to control fever, however, it can induce adverse events, such as acute liver failure (84). In our study, four downregulated genes (*Cyp1a2*, *Cyp2e1*, *Cyp1a1,* and *Sult2a1*) were involved in the pharmacokinetic of acetaminophen. The downregulation of metabolic transcripts are alleviated in infected livers at 18 dpi (**Figure 3**), indicating that downregulation of metabolic processes in infected liver varies by stage of infection and that acute *T. gondii* infection causes more inhibition of the hepatic metabolic processes. This result is consistent with previous work showing downregulation of genes involved in liver metabolism following *T. gondii* infection (72, 73).

Downregulation of xenobiotics or drug metabolism in liver is related to inflammatory response (85). Although transcripts in the cluster showing higher expression in most infected tissues were significantly enriched in immune-related terms and pathways (**Supplementary Table 5**), transcripts in the high expression pattern in the liver cluster (**Figure 2A**) were also significantly enriched in terms related to inflammatory processes (**Supplementary Table 6**). This suggests that liver exhibited more inflammatory response than other tissues. Likewise, transcripts in the cluster with high expression pattern at 18 dpi were significantly enriched in GO terms related to immune responses (**Supplementary Table 7**). The ability of *T. gondii* to cause severe hepatic pathologies has been demonstrated (86, 87), and it is possible that the prominent inflammatory response observed in our study contributes to the pathologies observed in infected livers.

CONCLUSION

RNA-seq was used to determine the global changes in the porcine transcriptome subsequent to *T. gondii* infection at 6 and 18 days post infection. Hundreds of DETs exhibited differential expression profiles in infected tissues and were clustered into five expression patterns. Infection induced downregulation of various metabolic processes in most infected tissues, especially in the liver during acute infection. The WGCNA analysis showed that, *T. gondii* infection causes differential expression of transcription factors, such as Irf1, Irf8, Stat1, and Stat3. We also identified 45 DETs encoding detoxifying enzymes involved in the metabolism of 300 xenobiotics or drugs. These data improve our understanding of the molecular changes that occur during *T. gondii* infection in pigs. Although results obtained in pigs may not be readily transferrable to humans, given the physiological and immunological similarities between pigs and humans, our findings may facilitate the understanding of how humans might respond to *T. gondii* infection.

ETHICS STATEMENT

The study design was reviewed and approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences (CAAS). The procedures involving animals were carried out in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. Animals were monitored every day for the development of clinical signs of toxoplasmosis. All efforts were made to minimize suffering and to reduce the number of pigs used in the experiment.

AUTHOR CONTRIBUTIONS

X-QZ, HE, and J-JH conceived and designed the study and critically revised the manuscript. J-JH performed the experiment, analyzed the transcriptomic data, and drafted the manuscript. JM and HE helped in study design, implementation, data analysis, and manuscript revision. J-LW, F-KZ, J-XL, B-TZ, and Z-XW helped in the study implementation. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01531/full#supplementary-material

Supplementary Figure 1 | Sequencing qualities of the uninfected and infected tissues at 6 dpi. The darker the color the better the global sequencing quality.

Supplementary Figure 2 | Sequencing qualities of the uninfected and infected tissues at 18 dpi. The darker the color the better the global sequencing quality.

Supplementary Figure 3 | Distribution of sequencing qualities. Vertical axis represents the percentage of clean reads with sequencing quality > Q20. Horizontal axis represents the samples sequenced in the present study.

Supplementary Figure 4 | Scale-free topology fit index of coexpression analysis. Scale independence map shows the relationship between soft power and scale free topology model fit of WGCNA analysis. Mean connectivity map shows the relationship between soft power and mean connectivity which summarizes the connection strengths with other genes.

Supplementary Table 1 | Primers used for quantitative reverse transcriptase qRT-PCR assay to validate the RNA-seq data.

Supplementary Table 2 | Normalized *Toxoplasma gondii* DNA load in infected pig tissues.

Supplementary Table 3 | Differential expression profiles of transcripts across various tissues of the pigs.

Supplementary Table 4 | Significantly enriched GO terms and pathways of DETs in the lower expression cluster in most infected tissues.

Supplementary Table 5 | Significantly enriched GO terms and pathways of DETs in the higher expression cluster in most infected tissues.

Supplementary Table 6 | Significantly enriched GO terms and pathways of DETs in the higher expression cluster in infected liver.

Supplementary Table 7 | Significantly enriched GO terms and pathways of DETs in the higher expression cluster at 18 dpi.

Supplementary Table 8 | Modules of coexpressed genes.

Supplementary Table 9 | Details of the host genes significantly correlated with *T. gondii* load (HGSCTG).

Supplementary Table 10 | Relationship between 330 xenobiotics and differentially expressed metabolism related genes.

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The Multitasking *Fasciola gigantica* Cathepsin B Interferes With Various Functions of Goat Peripheral Blood Mononuclear Cells *in vitro*

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Cathepsin B, a lysosomal cysteine protease, is thought to be involved in the pathogenesis of *Fasciola gigantica* infection, but its exact role remains unclear. In the present study, a recombinant *F. gigantica* cathepsin B (rFgCatB) protein was expressed in the methylotrophic yeast *Pichia pastoris*. Western blot analysis confirmed the reactivity of the purified rFgCatB protein to serum from *F. gigantica*-infected goats. The effects of serial concentrations (10, 20, 40, 80, and 160 μg/ml) of rFgCatB on various functions of goat peripheral blood mononuclear cells (PBMCs) were examined. We demonstrated that rFgCatB protein can specifically bind to the surface of PBMCs. In addition, rFgCatB increased the expression of cytokines (IL-2, IL-4, IL-10, IL-17, TGF-β, and IFN-γ), and increased nitric oxide production and cell apoptosis, but reduced cell viability. These data show that rFgCatB can influence cellular and immunological functions of goat PBMCs. Further characterization of the posttranslational modification and assessment of rFgCatB in immunogenicity studies is warranted.

Keywords: ***Fasciola gigantica*, cysteine protease, cathepsin B, immunomodulation, host-parasite interaction**

INTRODUCTION

Fasciolosis, caused by the liver flukes *Fasciola gigantica* (*F. gigantica*) and *Fasciola hepatica* (*F. hepatica*), is an important parasitic disease with a worldwide distribution (1). These liver flukes can infect a wide range of mammalian species, including livestock, wild animals, and humans. *F. gigantica* is responsible for significant economic losses in the buffalo-producing countries due to its chronic morbidity and adverse effects on the animal health, fecundity, and productivity (2). Adding to the challenge is the emerging evidence of *F. gigantica* resistance against albendazole and rafoxanide (3), and the lack of a commercial vaccine. Better understanding of the mechanisms and factors that shape the immuno-pathogenesis of fasciolosis may ultimately facilitate the design of new immunotherapeutic strategies for efficient treatment of fasciolosis.

Fasciola spp. employ multiple strategies to evade the host immune response using various molecules in their excretory/secretory (E/S) products (4–7), such as cathepsin B and L proteases (8–10). At least six types of cathepsin B have been detected in the immature and invasive stages

of *Fasciola*, and have been shown to play roles in the pathogenesis of fasciolosis (9, 11, 12). Due to their immune-modulatory functions, cysteine proteases such as cathepsin B have attracted significant attention as potential immuno-therapeutic targets to control liver fluke infection (9).

In *F. hepatica*, cathepsins interact with host immune cells and skew the immune response toward a non-protective Th2-mediated/regulatory response (13). In *F. gigantica*, cathepsin B2 and B3 digest host substrates, such as immunoglobulin, fibronectin, and collagen (14–16). Also, cathepsin B5, expressed in immature and adult stages of *F. gigantica*, can digest host proteins (17). The recombinant proteins (rFgCatB2 and rFgCatB3) can elicit a mixed Th1/Th2 immune response with the predominance of Th2 cytokines (16). Despite significant efforts, information about the modulatory effects of *F. gigantica* cathepsin B on the host innate immune cells is still limited.

In the present study, the gene encoding *F. gigantica* cathepsin B was cloned and expressed in *Pichia pastoris*. We characterized the modulatory effects of the purified recombinant *F. gigantica* cathepsin B protein (rFgCatB) on various functions of goat peripheral blood mononuclear cells (PBMCs), including cytokine secretion, cell viability, nitric oxide (NO) production, and apoptosis. We show that rFgCatB induces a mixed Th1/Th2/Th17 immune response and significantly influences other functions of goat PBMCs. Our findings demonstrate the feasibility of including rFgCatB protein in a vaccination trial against fasciolosis.

MATERIALS AND METHODS

Ethics Statement

All experimental protocols were reviewed and approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permit No. 2018-012). All animal experiments were performed in strict compliance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. All efforts were made to minimize the suffering of animals, and daily health checks were performed during the entire experiments.

Animals and Collection of Blood Cells

Eight local crossbred goats (4- to 7-month-old) were obtained from Laboratory Animal Center of Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Science. All goats were kept in-door and dewormed with albendazole and ivermectin tablets (Xining Fengyuan Agricultural and Animal Sci-Tech Company, Xining, China) to eliminate any potential existing helminth infection. Before and 2 weeks after treatment, fecal samples from each goat were microscopically examined for helminth eggs. This analysis showed that all goats used in the study are free from any prior or current helminth infection. For the production of antisera, four female New Zealand rabbits (3-month-old) were purchased from Laboratory Animal Center of Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Science and were housed under specific-pathogen-free conditions, with access to food and water *ad libitum*. Peripheral venous blood samples were collected from three healthy goats and peripheral blood mononuclear cells (PBMCs) and monocytes were isolated and cultured as previously described (7).

Parasite Preparation

Adult flukes were harvested from the gall bladder of naturally infected buffaloes at local slaughterhouses in Guangxi Zhuang Autonomous Region, PR China. The harvested flukes were washed with phosphate buffered saline (PBS, pH7.4) and immediately used for RNA isolation or stored at −80°C with RNA stabilizer for future use. The flukes were identified as *F. gigantica* based on amplification and sequencing of the internal transcribed spacer 2 (ITS-2) of the ribosomal DNA (18). Sequence alignment showed no difference between the ITS-2 sequence obtained in our study and the ITS-2 sequence obtained previously from *F. gigantica* samples collected from buffaloes in Guangxi province (GenBank accession No. AJ557569).

Cloning and Characterization of *FgCatB* Gene

Due to the lack of genomics data on *F. gigantica*, we have searched *F. hepatica* E/S product's dataset produced by liquid chromatography-tandem mass spectrometry (LC-MS/MS), *F. hepatica* cDNA library available from previous proteomic studies, and the BLASTx search protein database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), in order to identify homologous cathepsin B protein sequences. This analysis identified *F. hepatica* cathepsin B protein isoform (FhCatB) sequence (GenBank accession No. Z22768.1), which was used to design primers to amplify *F. gigantica* cathepsin B (*FgCatB*) gene sequence. Total *F. gigantica* RNA was isolated from 30 mg of adult *F. gigantica* flukes using Trizol reagent (Invitrogen, San Diego, USA). The first-strand cDNA was synthesized by reverse transcription polymerase chain reaction (RT-PCR) using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, (EU) Lithuania). The cDNA was used as a template to amplify *FgCatB* gene using two oligonucleotide primers: 5′-CCG GAA TTC CAT ATG AGC TTA CTG ATC TCC AGC-3′ (forward) and 5′- ATT TGC GGC CGC CTC GAG TTG GGG TAA TTT TGG C-3′ (reverse). The oligonucleotide primers were synthesized with the *EcoRI* (forward) or *Not* I (reverse) restriction site underlined. The resulting amplified *FgCatB* gene product was digested with *Eco*R I and *Not* I, and cloned into pMD19-T (Takara, Dalian, Liaoning, China). The recombinant plasmid was transformed into *Trans5α* chemically competent cells (TransGen Biotech, Beijing, China). Several positive clones were selected and sequenced by GenScript (Nanjing, Jiangsu, China) to confirm the correct insertion/orientation of *FgCatB* gene in the vector. The signal peptide, transmembrane helices (TMHs) and *N*-glycosylation sites of the *FgCatB* sequence were predicted using SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/), TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively.

Expression of rFgCatB Protein

A single positive clone containing the *FgCatB* gene was selected and the *FgCatB* gene fragment was sub-cloned into pPIC9K vector. A carboxyl-terminal His6 tag and appropriate restriction sites were included in the expression plasmid to enable purification. The plasmid designated as pPIC9K-*FgCatB* was linearized with *Sal* I and electroporated into the methylotrophic yeast *P. pastoris* GS115 strain using a GenePulser X cell TM (Bio-Rad, Hercules, California, USA). Positive recombinant *P. pastoris* clones containing the insert were selected for expression by inoculating into 15 ml of buffered complex medium containing glycerol (BMGY). The inoculated BMGY medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 1% [wt/vol] yeast nitrogen base, 1% [wt/vol] glycerol, 0.00004% [wt/vol] biotin, and 0.1 M potassium phosphate [pH 6.0]) in 100 ml conical flasks was incubated at 28°C with vigorous shaking for 24 h. The cells were harvested by centrifugation (250 × g for 10 min), resuspended in 20 ml of buffered complex medium containing methanol (BMMY; BMGY medium with 1% methanol substituted for glycerol). The culture was allowed to continue growing for 4 days. During *FgCatB* gene expression induction period, methanol was added every 24 h to maintain a final concentration of 1% (v/v). The cells were pelleted by centrifugation (2,500 × g at 4°C for 10 min) and the culture supernatant was harvested for protein extraction.

Purification of Recombinant *F. gigantica* Cathepsin B (rFgCatB) Protein

The yeast culture supernatant containing rFgCatB protein was concentrated by centrifugation at 4,000 × g for 15 min using Amicon® Ultra 10 K centrifugal filter device. The concentrated supernatant was purified using the His GaviTrap Kit (GE Healthcare, Buckinghamshire, UK) at 4°C. The rFgCatB protein was eluted with elution buffer (20 mM PBS, 0.5 M NaCl, 500 mM imidazole, PH 7.4) and dialyzed against 1×PBS to remove imidazole. The concentration of the protein was determined by the Bradford method, using bovine serum albumin (BSA) as the standard. Purified proteins were stored at −80°C until further analysis.

Preparation of Antibodies

Four, 4- to 7-month-old, goats were challenged orally with 250 viable encysted metacercariae of *F. gigantica*. After 3 months, the goat sera containing anti-*F. gigantica* antibodies were collected. Serum was collected from one healthy naïve goat (negative control) and stored frozen at −80°C. Specific antibodies against rFgCatB protein were produced by immunizing three New Zealand rabbits with rFgCatB. For primary immunization, 200 μg of the purified rFgCatB protein mixed with complete Freund's adjuvant (1:1) were injected subcutaneously into multiple sites at the back of the rabbits, followed by four booster doses with 100 μg of the recombinant protein in incomplete Freund's adjuvant at 2-week intervals. One week after the last injection, antisera against rFgCatB was collected. In the meantime, serum was collected from one healthy rabbit (negative control) and stored frozen at −80°C.

SDS-PAGE and Western Blotting

The isolated protein (20 μg) was separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels and stained with Coomassie Blue. The protein migrated on gels as a "blurred" smear without showing the expected band size, indicating that rFgCatB is a glycoslyated protein. Therefore, rFgCatB was deglycosylated under denaturing conditions using Protein Deglycosylation Mix II (New England Biolab® Inc., USA), as per the manufacturer's instructions. The deglycosylated rFgCatB protein was resolved on 12% SDS-PAGE gels, followed by Coomassie Blue staining. Also, the deglycosylated rFgCatB was transferred onto Hybond-C extra nitrocellulose membrane (Amersham, London, UK). The membrane was blocked using 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at ambient temperature, followed by incubation with primary antibodies (antiserum from goats experimentally infected with *F. gigantica*) for 12 h at 4°C (1:100 in TBST). After being washed three times (5 min each) with TBST, the membrane was incubated with HRP-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO, USA) for 1 h at 37°C (1:2500 in TBST). Finally, freshly prepared 3,3′-diaminobenzidine (DAB, Sigma) was used as a chromogenic substrate to visualize the immunoreaction.

Measurement of rFgCatB Activity

The enzyme activity of rFgCatB was measured using Cathepsin B Activity Assay Kit (Abcam, ab65300) according to the manufacturer's instructions. Briefly, 50 μg of rFgCatB protein was adjusted to 50 μL per well with cell lysis buffer for experimental samples in a 96-well plate. Fifty microliters of blank cell lysis buffer were used for measuring background. Next, 50 μL CB Reaction Buffer followed by 2 μL of cathepsin B substrate Ac-RR-AFC (amino-4-trifluoromethyl coumarin) were added to each well. The plates were incubated at 37°C for 2 h protected from light, and fluorescence from the cathepsin B-cleaved substrate was measured at excitation/emission (Ex/Em) = 400/505 nm using a fluorescent microplate reader (Thermo scientific, Varioskan LUX Multimode Microplate Reader). The relative enzyme activity of rFgCatB was represented as the fold increase in the fluorescence intensity compared with the cathepsin B inhibitor-treated control.

Immunofluorescence Detection of rFgCatB Protein Binding to Goat PBMCs

Goat PBMCs were incubated with 10 μg/ml of rFgCatB in a humidified atmosphere of 5% CO_2 at 37°C for 1 h. The rFgCatB-treated cells were fixed with 4% paraformaldehyde at ambient temperature for 15 min, washed three times in PBS (5 min each), and subsequently treated with blocking solution (4% BSA in PBS) for 1 h to minimize background staining. rFgCatB-treated or non-treated control PBMCs were incubated with rabbit anti-rFgCatB antibody (dilution, 1:100) for 12 h at 4°C and washed three times in PBS (5 min each). Cells were stained with Cy3 conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:500) (Beyotime, Haimen, Jiangsu, China) for 1 h at 37°C. Hoechst 33342 (Invitrogen, Eugene, Oregon, USA) was used to stain the nucleus. Localization of rFgCatB was visualized using a Zeiss laser scanning confocal microscope (LSM710, Zeiss, Jena,

Germany) at 100 × magnification and images were analyzed using Zen 2012 imaging software.

Cytokine Analysis

The concentrations of cytokines were evaluated in the supernatant of 5×10^4 PBMCs seeded into 24-well tissue culture plates in 1 ml RPMI 1640 medium/well. Serial concentrations (10, 20, 40, 80, and 160 μg/ml) of rFgCatB protein or equal volume of PBS (control) were added to the wells. The culture plates were incubated at 37°C with 5% CO_2 for 72 h. The supernatants were collected and the concentrations of interleukin-2 (IL-2), IL-4, IL-10, IL-17, interferon gamma (IFN-γ), and transforming growth factor-beta (TGF-β) were determined using goat enzyme linked immunosorbent assay (ELISA) kits (Mlbio, Shanghai, China) as per the manufacturer's instructions.

The Effect of rFgCatB Protein on Cell Viability

The effect of rFgCatB protein on the viability of PBMCs was examined by a CCK-8 assay (Beyotime, Haimen, Jiangsu, China). This assay is based on the measurement of the reduction of a water-soluble tetrazolium salt WST-8 by dehydrogenases in viable cells. Briefly, PBMCs (10^4 cells/100 μl RPMI 1640 medium/well) seeded into 96-well tissue culture plates were incubated with serial concentrations (10, 20, 40, 80, or 160 μg/ml) of rFgCatB protein or equal volume of PBS (control) at 37°C in a humidified atmosphere with 5% CO_2. Following 48 h incubation, 10 μl of CCK-8 reagent were added per well and the culture plates were further incubated under the same conditions for 4 h in protected from light. The optical density at 450 nm (OD_{450}) was measured using a microplate reader (Bio-Rad, Hercules, California, USA). The OD_{450} of control wells (cells incubated with PBS) was set as 100% and the cell viability index was calculated using the formula: OD_{450} rFgCatB /OD_{450} control.

Determination of Nitric Oxide (NO)

PBMCs were seeded into a 24-well tissue culture plate at 5×10^4 cells/well in 1 ml RPMI 1640 medium. Cells were incubated with various concentrations (10, 20, 40, or 80 μg/ml) of rFgCatB protein or equal volume of PBS (control) at 37°C with 5% CO_2 for 24 h. The NO level in PBMC culture supernatant was determined by measuring the concentrations of nitrite using the Total Nitric Oxide Assay Kit (Beyotime, Haimen, Jiangsu, China). A microplate reader (Bio-Rad, Hercules, California, USA) was used to measure the absorbance values at 540 nm (OD_{540}). NO levels were calculated using a standard curve generated by 0 to 80 μM/L sodium nitrites.

Evaluation of the Apoptotic Effect of rFgCatB Protein

Flow cytometry analysis (BD Biosciences, San Jose, California, USA) was carried out to evaluate the apoptosis in PBMCs using the Annexin V-FITC kit (Beyotime, Haimen, Jiangsu, China). PBMCs seeded into a 24-well tissue culture plate at 5×10^4 cells/well in 1 ml RPMI 1640 medium were incubated with the above mentioned concentrations of rFgCatB protein or equal volume of PBS (control) at 37°C with 5% CO_2 for 24 h. Then, cells were washed twice with PBS, re-suspended in binding buffer, and stained with Annexin V and Propidium Iodide (PI) according to the manufacturer's instructions. The results were analyzed using FlowJo 10.

Statistical Analysis

The statistical analyses were performed by one-way ANOVA, followed by a Dunnett's test or *t*-test using GraphPad Premier 6.0 software package (GraphPad Prism, San Diego, California, USA). Significant differences between rFgCatB-treated and control groups are indicated in the figures by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ or ****, $P < 0.0001$). Data were presented as means ± standard deviation (SD). All experiments were repeated at least three separate times.

RESULTS

Identification, Cloning, and Expression of rFgCatB Protein

We performed multiple searches to identify homologous of *F. gigantica* cathepsin B sequence in the genome of *F. hepatica*. This analysis identified *F. hepatica* cathepsin B-like protease (GenBank accession no. Z22768.1) sequence, which was used to design 5′ and 3′ primers to amplify the *FgCatB* gene. The cDNA fragment of *FgCatB* was successfully cloned into the pMD19-T cloning vector and the positive pMD19-T-*FgCatB* clones were subjected to nucleic acid sequencing. The obtained *FgCatB* sequence has been submitted to GenBank under accession number MN038412. The amino acid sequence similarity search showed that cathepsin B endopeptidase of *F. hepatica* (THD22097.1) has the highest similarity (100% homology) to *FgCatB*. The ORF contained 1,038 base-pair (bp) and encoded 345 amino acids. The deduced amino acid sequence predicts the existence of a signal peptide, two *N*-linked glycosylation sites and four protein kinase C phosphorylation sites, however, no TMH was detected.

SDS-PAGE and Western Blotting Analysis

To verify the presence of FgCatB protein in *F. gigantica*-derived material, *FgCatB* gene fragment was cloned into the pPIC9K vector and the positive clones, designated as pPIC9K-*FgCatB*, were transformed into *P. pastoris*. The recombinant protein (rFgCatB) was successfully isolated from the culture supernatant of *P. pastoris*. The expected molecular mass of rFgCatB is 38.2 kDa, however after 72 h of induction with 1% methanol the purified protein exhibited a heterogeneous molecular mass ranging from ∼36–70 kDa on SDS-PAGE. Two bands of approximately 38 and 36 kDa appeared after deglycosylation using endoglycosidase H (Endo H) treatment, which cleaves high-mannose *N*-linked glycans (**Figure 1A**). Western blot analysis using serum from *F. gigantica*-infected goats confirmed the specificity of the two bands, which were absent when the Western blot was probed with serum from healthy goats (**Figure 1B**).

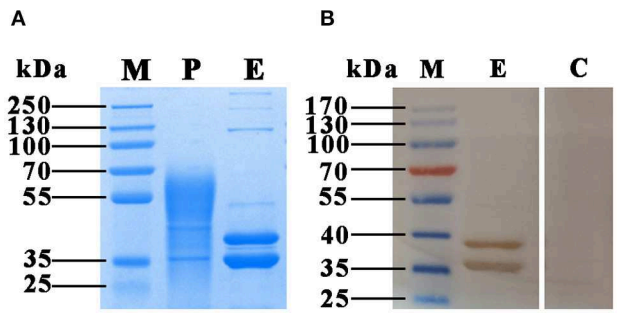

FIGURE 1 | SDS-PAGE and Western blotting analysis of the rFgCatB protein purified from the culture supernatant of *P. pastoris*. **(A)** Proteins were resolved on 12% acrylamide gels and stained with Coomassie brilliant blue R250. Lane M, protein molecular weight marker; Lane P, purified rFgCatB appeared heterogeneous and ranged in size from ~36 to 70 kDa; Lane E, rFgCatB treatment with endoglycosidase H (Endo H) revealed two distinct bands at ~38 and 36 kDa. **(B)** The protein treatment with Endo H was run under non-reducing conditions, and visualized by immunodetection using specific antibodies and enhanced chemiluminescence. Lane M, protein molecular weight marker; Lane E, was loaded with rFgCatB digested with Endo H. Serum from *F. gigantica*-infected goats detected ~ 38 and 36 kDa bands; Lane C, was loaded with rFgCatB, which did not react with serum of healthy goats.

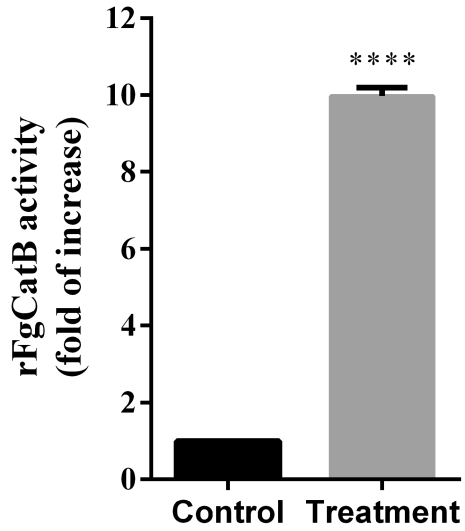

FIGURE 2 | *Fasciola gigantica*-derived rFgCatB enzymatic activity was determined by examining its ability to cleave the fluorescent synthetic substrate RR-AFC to release free AFC. The enzyme activity of rFgCatB was measured by Cathepsin B Activity Assay Kit as described in the materials and methods and the result showed a high activity of the rFgCatB (*t*-test, $t = 72.68$, $P < 0.0001$). Asterisks indicate statistical significance between rFgCatB sample and cathepsin B inhibitor-treated control sample (****$P < 0.0001$).

Enzymatic Activity of rFgCatB

The activity of cathepsin B was examined using the Fluorometric ab65300 assay kit. *Fasciola gigantica*-derived rFgCatB enzymatic activity was determined by measuring its ability to cleave the fluorescent synthetic substrate RR-AFC to release free AFC. The results showed that the enzyme activity of rFgCatB is several fold higher than that of the control, confirming the functional activity for cathepsin B (**Figure 2**).

Binding Affinity of rFgCatB Protein to Goat PBMCs

Indirect immunofluorescence staining was used to determine the binding affinity of rFgCatB protein to the surface of PBMCs. By incubating rFgCatB-treated PBMCs with rabbit anti-rFgCatB antibodies, the localization of the red Cy3 conjugated goat anti-rabbit IgG secondary antibody on the cell surface was observed, suggesting successful binding of rFgCatB to the surface of PBMCs (**Figure 3**). There was no fluorescence observed in the untreated control cells.

rFgCatB Protein Increased Cytokine Production

To understand how rFgCatB modulates cytokine production of PBMCs, the levels of six cytokines, IL-2, IL-4, IL-10, IL-17, IFN-γ, and TGF-β, were determined. As shown in **Figure 4**, when PBMCs were treated with serial concentrations of rFgCatB protein, the production of all six cytokines was significantly increased compared with control (PBS-treated) PBMCs.

Cytotoxic Effect of rFgCatB Protein

We examined whether rFgCatB protein affects the viability of PBMCs. The CCK-8 assay showed that the viability of PBMCs was remarkably decreased following treatment with rFgCatB protein, at all tested protein concentrations (**Figure 5**).

Nitric Oxide (NO) Production

As shown in **Figure 6**, compared to the control (PBS-treated PBMCs), NO release was slightly increased in rFgCatB-treated PBMCs at 40 μg/ml and was significantly increased in rFgCatB-treated PBMCs at 80 μg/ml, but not at 10 or 20 μg/ml.

rFgCatB Protein Induced Cell Apoptosis

To explore whether rFgCatB protein induces apoptosis in goat PBMCs, Annexin V-FITC apoptosis assay was used. The rFgCatB protein significantly induced apoptosis in PBMCs at all tested concentrations compared to PBS-treated, control PBMCs (**Figure 7**). The apoptosis was induced in a dose-dependent manner with the percentage of apoptotic cells treated with rFgCatB at 10, 20, 40, and 80 μg/ml were 28.88 ± 2.631%, 30.95 ± 3.128%, 33.50 ± 2.152%, and 46.17 ± 5.955%, respectively.

DISCUSSION

In this study, we cloned and expressed the gene encoding cathepsin B of *F. gigantica* in the methylotrophic yeast *P. pastoris*. Although the expected size of the purified rFgCatB protein with six-histidine tag is 38.2 kDa, a significant increase in its molecular weight was observed. Yeast expression system has been known to introduce post-translational modifications such as glycosylation which may affect protein folding. Also, recombinant proteins produced in *P. pastoris* tend to be

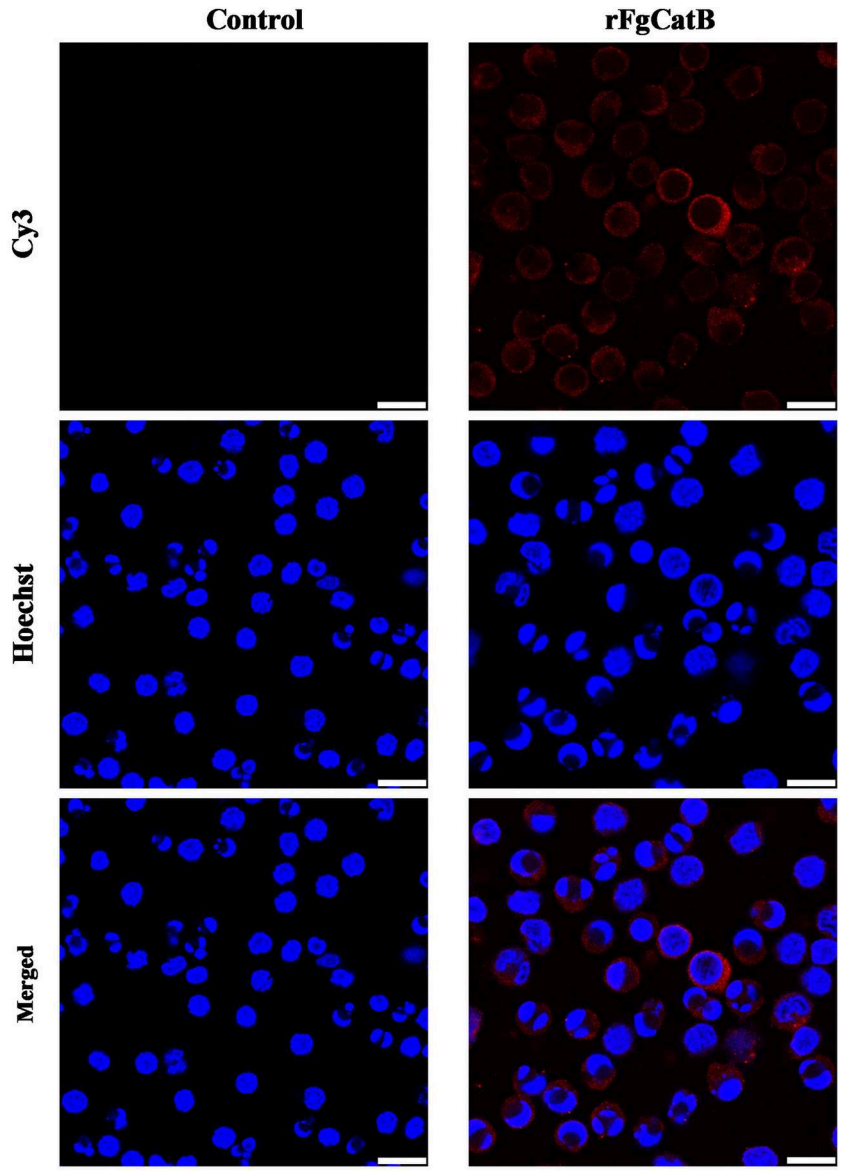

FIGURE 3 | Localization of *Fasciola gigantica*-derived rFgCatB protein on the surface of PBMCs. Cells were treated with rFgCatB and incubated with rabbit anti-rFgCatB primary antibody. Hoechst (blue) and Cy3-conjugated secondary antibody (red) were used to stain host cell nuclei and rFgCatB protein, respectively. Surface localization was observed in rFgCatB-treated cells, whereas no staining was detected in untreated (control) cells. Scale bars, 10 μm.

hypermannosylated. On SDS-PAGE gels, rFgCatB appeared as a group of bands with molecular weights between ∼36 and 70 kDa. The observed heterogeneity in the size of the protein may be attributed to *N*-linked glycosylation (19). Deglycosylation of rFgCatB using Endo H revealed ∼38 kDa band, which corresponds to the theoretical molecular mass of rFgCatB, and another band with molecular masse of 36 kDa, suggesting the presence of two different glycosylated species (i.e., diglycosylated forms) of rFgCatB, particularly, as both reacted in Western blot. This type of finding has been also reported for cathepsin B from the Asiatic liver fluke *Opisthorchis viverrini* (20).

Our results showed that rFgCatB induced expression of Th1 type cytokines (IL-2 and IFN-γ), Th2 type cytokines (IL-4, IL-10, and TGF-β), and Th17 type cytokine (IL-17), suggesting that rFgCatB can induce a mixed T helper 1 (Th1)-, Th2-, and Th17-type immune response. The high levels of pro-inflammatory cytokines (IL-2 and IFN-γ) and activation of monocytes have been associated with intestinal pathology and release of NO to limit the fluke growth (21–23). On the other hand, high expression of Th2 anti-inflammatory cytokines can facilitate parasite persistence, while minimizing host tissue damage (24–27). For example, the anti-inflammatory cytokine IL-4 inhibits

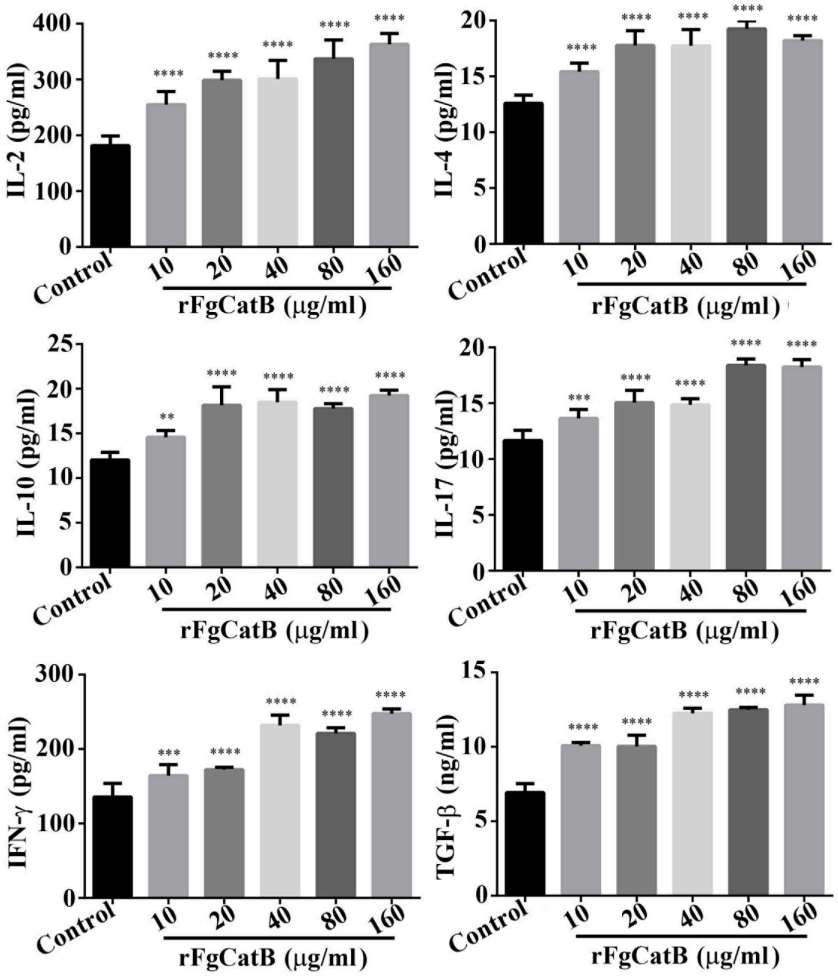

FIGURE 4 | rFgCatB protein stimulated the production of cytokines. Goat PBMCs were incubated for 72 h in the presence of PBS or serial concentrations of rFgCatB protein. The levels of cytokines in the supernatant of cultured PBMCs were quantified by ELISA. Results showed that rFgCatB induced the expression of all examined cytokines in a dose-dependent manner. Graphs represent means ± standard deviations of data from three independent biological replicates (IL-2 (10 μg/ml: $F_{(5,36)} = 46.98$, $P < 0.0001$; 20 μg/ml: $F_{(5,36)} = 46.98$, $P < 0.0001$; 40 μg/ml: $F_{(5,36)} = 46.98$, $P < 0.0001$; 80 μg/ml: $F_{(5,36)} = 46.98$, $P < 0.0001$; 160 μg/ml: $F_{(5,36)} = 46.98$, $P < 0.0001$), IL-4 (10 μg/ml: $F_{(5,36)} = 43.65$, $P < 0.0001$; 20 μg/ml: $F_{(5,36)} = 43.65$, $P < 0.0001$; 40 μg/ml: $F_{(5,36)} = 43.65$, $P < 0.0001$; 80 μg/ml: $F_{(5,36)} = 43.65$, $P < 0.0001$; 160 μg/ml: $F_{(5,36)} = 43.65$, $P < 0.0001$), IL-10 (10 μg/ml: $F_{(5,36)} = 40.51$, $P = 0.0012$; 20 μg/ml: $F_{(5,36)} = 40.51$, $P < 0.0001$; 40 μg/ml: $F_{(5,36)} = 40.51$, $P < 0.0001$; 80 μg/ml: $F_{(5,36)} = 40.51$, $P < 0.0001$; 160 μg/ml: $F_{(5,36)} = 40.51$, $P < 0.0001$), IL-17 (10 μg/ml: $F_{(5,36)} = 77.39$, $P = 0.0002$; 20 μg/ml: $F_{(5,36)} = 77.39$, $P < 0.0001$; 40 μg/ml: $F_{(5,36)} = 77.39$, $P < 0.0001$; 80 μg/ml: $F_{(5,36)} = 77.39$, $P < 0.0001$; 160 μg/ml: $F_{(5,36)} = 77.39$, $P < 0.0001$), TGF-β (10 μg/ml: $F_{(5,36)} = 137.5$, $P < 0.0001$; 20 μg/ml: $F_{(5,36)} = 137.5$, $P < 0.0001$; 40 μg/ml: $F_{(5,36)} = 137.5$, $P < 0.0001$; 80 μg/ml: $F_{(5,36)} = 137.5$, $P < 0.0001$; 160 μg/ml: $F_{(5,36)} = 137.5$, $P < 0.0001$), and IFN-γ (10 μg/ml: $F_{(5,36)} = 96.82$, $P = 0.0003$; 20 μg/ml: $F_{(5,36)} = 96.82$, $P < 0.0001$; 40 μg/ml: $F_{(5,36)} = 96.82$, $P < 0.0001$; 80 μg/ml: $F_{(5,36)} = 96.82$, $P < 0.0001$; 160 μg/ml: $F_{(5,36)} = 96.82$, $P < 0.0001$). Asterisks indicate statistical significance between treated and untreated control PBMCs (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ compared with control).

NO production (28) and promotes Th2 differentiation (29), thereby facilitating the production of other anti-inflammatory cytokines (e.g., IL-10 and TGF-β) and inhibiting pro-inflammatory cytokines (e.g., IL-2 and IFN-γ) (30, 31). Also, IL-10 decreases the production of IFN-γ and IL-2 (22, 31).

TGF-β, together with other inflammatory cytokines, can promote Th17 differentiation (32–34). Th17 cells play an important role in host protection against various parasitic infections by recruiting macrophages and neutrophils to infected tissues, and through the modulation of Th1/Th2 balance (34–36). The role of IL-17 in the inflammatory process during *F. gigantica* infection has been reported (37, 38). Interestingly, TGF-β can inhibit T cell proliferation by suppressing the production of IL-2, and inhibiting the differentiation of Th1 and Th2 cells (39).

We have previously shown that *F. gigantica* proteins, rFg14-3-34 and rFgRab10, inhibit cell proliferation, and induce apoptosis and NO production in goat PBMCs (6, 7). The results of the present study lend further support to these previous findings, where rFgCatB was found to bind to the surface of PBMCs similar to what we have demonstrated for rFg14-3-4 and rFgRab10 proteins (6, 7), and to reduce the viability

and increase apoptosis of PBMCs. The biological relevance of the pro-apoptotic effect of rFgCatB on PBMCs remains to be determined. However, induction of apoptosis, rather than necrosis, may favor the parasite's persistence because apoptotic cell death does not provoke inflammatory response (40), which can be detrimental to the parasite's survival inside the host.

The antiproliferative and pro-apoptotic effects of E/S products of *Fasciola* spp. on immune cells are some of the strategies used by these liver flukes to hamper immune defenses, leaving the host more vulnerable to infection. *F. hepatica*-derived E/S products have been shown to inhibit the proliferation of sheep lymphoid cells, especially $CD4^+$ T lymphocytes (41–43), reduce the proliferation of rat spleen mononuclear cells (44) and induce apoptosis of murine

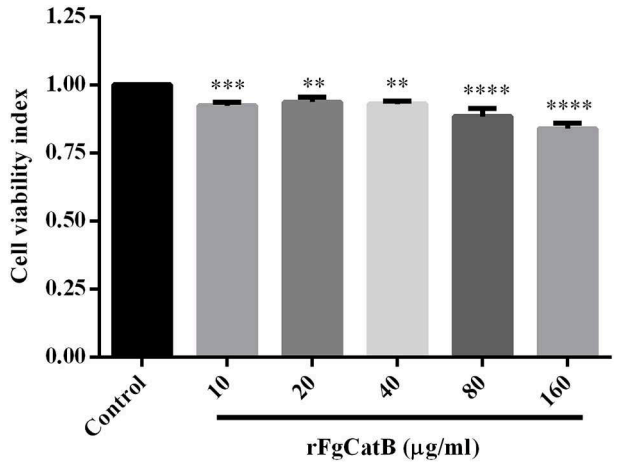

FIGURE 5 | Effect of rFgCatB protein on PBMC viability. Goat PBMCs were treated with PBS or with serial concentrations of rFgCatB protein and incubated for 48 h at 37°C at 5% CO_2. Viability of cells was determined using CCK-8 assay. Results indicate that rFgCatB protein significantly reduced the viability of PBMCs in a dose-dependent manner. Graphs represent means ± standard deviations of data from three independent biological replicates (10 μg/ml: ANOVA, $F_{(5, 12)} = 28.50$, $P = 0.0007$; 20 μg/ml: ANOVA, $F_{(5, 12)} = 28.50$, $P = 0.0035$; 40 μg/ml: ANOVA, $F_{(5, 12)} = 28.50$, $P = 0.0015$; 80 μg/ml: ANOVA, $F_{(5, 12)} = 28.50$, $P < 0.0001$; 160 μg/ml: ANOVA, $F_{(5, 12)} = 28.50$, $P < 0.0001$). Asterisks indicate significant differences between rFgCatB-treated and PBS-treated control cells (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ compared with control).

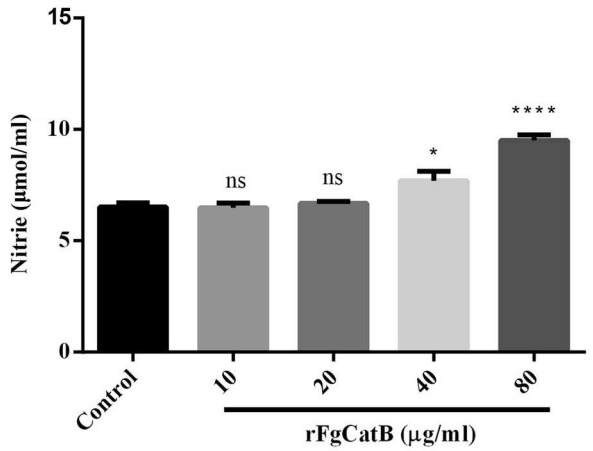

FIGURE 6 | Effects of rFgCatB protein on the production of NO. PBMCs were treated with PBS or with serial concentrations of rFgCatB protein and maintained at 37°C for 24 h. NO concentration was measured by Griess assay. Graphs represent means ± standard deviations of data from three independent biological replicates (10 μg/ml: ANOVA, $F_{(4, 10)} = 27.91$, $P = 0.9998$; 20 μg/ml: ANOVA, $F_{(4, 10)} = 27.91$, $P = 0.9702$; 40 μg/ml: ANOVA, $F_{(4, 10)} = 27.91$, $P = 0.0220$; 80 μg/ml: ANOVA, $F_{(4, 10)} = 27.91$, $P < 0.0001$). Asterisks indicate significant differences between rFgCatB-treated and PBS-treated control cells (*$p < 0.05$; ****$P < 0.0001$; ns, non-significant compared with control).

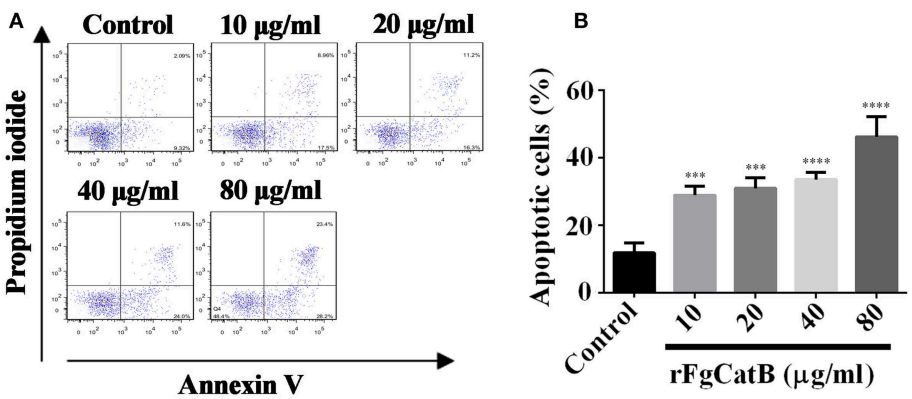

FIGURE 7 | rFgCatB protein induced apoptosis in goat PBMCs. Annexin V/PI staining was used to quantify apoptotic cells by flow cytometry. **(A)** The FACS plot showing apoptosis of PBMCs in response to exposure to rFgCatB protein. **(B)** Apoptotic cells (Annexin V+/PI-) were plotted and compared with the percentage of cell population. Graphs represent means ± standard deviations of data from three independent biological replicates (10 μg/ml: ANOVA, $F_{(4, 10)} = 34.92$, $P = 0.0006$; 20 μg/ml: ANOVA, $F_{(4, 10)} = 34.92$, $P = 0.0003$; 40 μg/ml: ANOVA, $F_{(4, 10)} = 34.92$, $P < 0.0001$; 80 μg/ml: ANOVA, $F_{(4, 10)} = 34.92$, $P < 0.0001$). The asterisks indicate significant differences between rFgCatB-treated and PBS-treated control goat PBMCs (***$P < 0.001$; ****$P < 0.0001$ compared with control).

eosinophils and peritoneal macrophages (45, 46). Also, immunosuppression of $CD4^+$ T lymphocytes has been observed in *F. hepatica*-infected goats (47). Additionally, *F. hepatica* can induce apoptosis in sheep PBMCs by up-regulating the expression of TNF-α and TNFR1/TNFR2 (48). The induction of apoptosis in sheep eosinophils (49) and peritoneal leucocytes (50) has been suggested to play a role in the pathogenesis of *F. hepatica* by supporting the survival of the juvenile parasites during the migratory and biliary stages of infection.

In summary, our data show that rFgCatB interacts with serum from goats infected with *F. gigantica* and accumulates at the surface of PBMCs. The importance of our data resides in the fact that rFgCatB represents a new mechanism for *F. gigantica* to evade the host's immune response through modulation of the immune response and biological functions of PBMCs. Exposure of these cells to rFgCatB caused increased production of cytokines (IL-2, IL-4, IL-10, IL-17, TGF-β, and IFN-γ), increased NO production, increased apoptosis, and inhibition of cell viability. Our data provide a proof of concept that rFgCatB is involved *F. gigantica*-interaction with immune cells. In the light of these findings and given that rFgCatB and other *F. gigantica*-derived proteins (e.g., rFg14-3-34 and rFgRab10) can modulate key cellular and immunological functions of goat PBMCs, future work should focus on identifying the appropriate synergistic combinations of these proteins to develop a cocktail vaccine for testing against *F. gigantica* infection.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

ETHICS STATEMENT

All experimental protocols were reviewed and approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permit No. 2018-012). All animal experiments were performed in strict compliance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. All efforts were made to minimize the suffering of animals, and daily health checks were performed during the entire experiments.

AUTHOR CONTRIBUTIONS

X-QZ, XL, and HE conceived the idea, planned the experiments, and provided critical feedback. DC performed the experiments, analyzed the data, and drafted the manuscript with the help of HE. A-LT, J-LH, J-XL, XT, and X-DY participated in the implementation of the study. All authors read and approved the final manuscript.

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IL-4 Mediated Resistance of BALB/c Mice to Visceral Leishmaniasis Is Independent of IL-4Rα Signaling via T Cells

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Previous studies infecting global IL-4Rα$^{-/-}$, IL-4$^{-/-}$, and IL-13$^{-/-}$mice on a BALB/c background with the visceralizing parasite *Leishmania donovani* have shown that the T helper 2 cytokines, IL-4, and IL-13, play influential but not completely overlapping roles in controlling primary infection. Subsequently, using macrophage/neutrophil-specific IL-4Rα deficient BALB/c mice, we demonstrated that macrophage/neutrophil unresponsiveness to IL-4 and IL-13 did not have a detrimental effect during *L. donovani* infection. Here we expand on these findings and show that CD4^{+} T cell-(Lck^{cre}), as well as pan T cell-(iLck^{cre}) specific IL-4Rα deficient mice, on a BALB/c background, unlike global IL-4Rα deficient mice, are also not adversely affected in terms of resistance to primary infection with *L. donovani*. Our analysis suggested only a transient and tissue specific impact on disease course due to lack of IL-4Rα on T cells, limited to a reduced hepatic parasite burden at day 30 post-infection. Consequently, the protective role(s) demonstrated for IL-4 and IL-13 during *L. donovani* infection are mediated by IL-4Rα-responsive cell(s) other than macrophages, neutrophils and T cells.

Keywords: ***Leishmania donovani*, IL-4Rα, IL-4, T cell, mice**

INTRODUCTION

Infection with the parasite *Leishmania donovani* causes visceral disease and can be fatal if it is not treated. Although there are major campaigns aimed at eliminating this disease e.g., World Health Organization 2020 roadmap, it still remains a serious neglected tropical disease (1) (https://www.who.int/leishmaniasis/en/), with no effective vaccine currently available (2). Successful pathogenesis is dependent on parasite survival in the host, a process mediated by a complex interplay of host factors. An in-depth understanding on the contribution of these factors to disease is therefore necessary to inform the development of novel or adjunct host-directed therapies (3, 4).

Earlier studies in this context revealed that the IFN-γ/IL-4 paradigm of resistance and susceptibility to intracellular infection, as defined for species causing cutaneous leishmaniasis (CL), does not apply holistically to species causing visceral leishmaniasis (VL). As with CL, protective immunity against this parasite relies on a Th1 response, which requires IL-12 production, and culminates in IFN-γ release (5, 6). In target tissues such as the liver, infection results in granuloma formation around infected macrophages (Kupffer cells) and eventual parasite death, primarily via the action of reactive nitrogen and oxygen intermediates (7, 8). However, unlike CL, a dominant inhibitory role for type 2 cytokines is less clear in murine models of VL (9). In asymptomatic and cured VL patients (10–12), both IFN-γ and IL-4-producing T cells have been identified and in the murine model of VL, protection is related to higher frequencies of cytokine-producing cells rather than altering the IFN-γ/IL-4 balance (13). In contrast, both human (12, 13) and murine (14) VL studies show that IL-10 is more important than IL-4 in immune suppression and parasite persistence.

Rather than being a detrimental cytokine for host protection, the evidence tends to suggest that type 2 immune responses may actually contribute to control of VL. Accordingly, our previous studies utilizing gene-deficient mice have identified protective roles for IL-4, IL-13, and IL-4Rα signaling during primary *L. donovani* infection (15–17). Control of parasite growth within the liver depends on the ability of Kupffer cells to clear parasites inside mature granulomas (15), a mechanism which requires T cell-derived IFN-γ (18) and the coordinated activity of macrophages which migrate toward the infected area. Enhanced susceptibility of IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-4Rα$^{-/-}$ mice to *L. donovani* infection was associated with a reduction in type 1 responses and retarded granuloma maturation so that fewer mature or sterile granulomas were present (15, 16, 19). In line with these observations, a recent study indicated that IL-10, and not IL-4, was responsible for manipulating monocytes/macrophages in VL infection (20). In addition to playing significant roles in controlling primary infection with *L. donovani*, IL-4, and IL-13 have also been associated with the successful outcome of sodium stibogluconate (SSG) treatment and vaccination with recombinant hydrophilic acylated surface protein (HASP)B-1 (15, 17, 19). While these studies added value to our understanding of the contribution of IL-4/IL-13 in host-protection against VL, they did not provide information on which cells were targeted by these cytokines via the IL-4Rα, and are therefore critical for protective immunity. Indeed, both IL-4 and IL-13 are pleiotropic cytokines and numerous cell types of both the innate and adaptive immune system produce and respond to these cytokines (21). In this regard, studies in cell-type specific IL-4Rα-deficient mice during CL has revealed a hierarchical interaction between the IL-4Rα chain and its ligands on different immune cells. To illustrate this, IL-4Rα signaling via DCs to produce IL-12 plays a protective role during cutaneous infection with *Leishmania major* (22), while IL-4Rα signaling via T cells (23) and Th2 induction, via macrophages and alternative activation (24), and via B cells and IL-4 production (25), all promote disease progression.

To further dissect the cell-specific requirements of IL-4/IL-13 signals on immune cells in VL, we used conditional cell-specific IL-4Rα deficient BALB/c mice, generated by the cre/*loxP* recombination system, to demonstrate that macrophage/neutrophil-specific (LysM) IL-4Rα signaling was not necessary for an effective healing response during VL, nor did it influence the outcome of SSG chemotherapy (16). Other possible target cells for IL-4 during VL may include dendritic cells (DC) (26, 27) and B cells (28) but more particularly CD4$^+$ (26, 29) and/or CD8$^+$ (30) T cells, whose active involvement has been shown not only to be essential to control primary infection and granuloma formation but also for successful SSG chemotherapy and therapeutic vaccination (15, 31, 32). Consequently, in this study we generated CD4$^+$ T cell-specific IL-4Rα$^{-/-}$ (Lck^{cre}IL-4Rα$^{-/lox}$) mice (23) and iLck^{cre}IL-4Rα$^{-/lox}$ mice that lack IL-4Rα on both CD4 and CD8 T cells (33) to determine the temporal role of IL-4 signaling via CD4$^+$ and CD8$^+$ T cells on the progression of VL infection. Unlike global IL-4Rα$^{-/-}$ mice infected with *L. donovani* that developed significantly higher parasite burdens than wild-type mice in this and previous studies (15), CD4$^+$ T cell specific IL-4Rα$^{-/-}$ mice were by comparison resistant to infection. Indeed, at day 30 post-infection CD4$^+$ T cell as well as pan T cell-specific IL-4Rα$^{-/lox}$ mice (iLck^{cre}IL-4Rα$^{-/lox}$) were more resistant than their wild-type littermate controls to hepatic infection with *L. donovani*. Increased susceptibility in global IL-4Rα$^{-/-}$ mice was associated with a diminished type 1 response and increased IL-10 production while CD4$^+$ T cell deficient IL-4Rα$^{-/lox}$ mice had comparable expression of IFN-γ on both CD4$^+$ and CD8$^+$ T cells and serum IL-10 levels similar to infected wild-type mice. Consequently, the protective effects of IL-4 during primary *L. donovani* infection are not mediated via direct effects on either CD4$^+$ or CD8$^+$ T cells, and IL-4 may even play a small regulatory role in these cells.

MATERIALS AND METHODS

Ethics Statement

Animal experiments and experimental procedures were carried out in line with UK Home Office regulations and the University of Strathclyde Animal Welfare and Ethical Review Board regulations under project license number: PPL 60/3525. BALB/c mice were all bred and maintained in the Biological Procedures Unit at the University of Strathclyde, Glasgow and experimental design and reporting adhere to the ARRIVE guidelines.

Animals and Parasites

Lck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice were created as described (23). Briefly, Lck^{cre}IL-4Rα$^{-/lox}$ mice were generated by inter-crossing IL-4Rα$^{lox/lox}$ BALB/c mice (34) with IL-4Rα$^{-/-}$ BALB/c mice (35) and BALB/c mice expressing the Cre-recombinase under the control of *Lck* locus, a T cell specific promoter (36) to generate hemizygous Lck^{cre}IL-4Rα$^{-/lox}$ mice. The efficiency of the deletion was characterized (23) whereby Lck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice have IL-4Rα selectively deleted from CD4$^+$ T cells (CD4$^+$ T cell specific IL-4Rα deficient mice). As these mice have variable and incomplete deletion in CD8$^+$ T cells (28), mice

lacking the IL-4Rα on all T cells (iLck^{cre}IL-4Rα$^{-/lox}$ mice) were produced as described (33). The iLck^{cre} construct was designed with a nuclear localization signal and an eukaryotic translation start site at the 5' end of the *Cre*-recombinase, which meant that the insert integrated downstream from the *Lck* proximal promoter (33, 37). Non-T cell populations such as B cells, macrophages and DC express IL-4Rα as normal. PCR genotyping studies were used to identify Lck^{cre}IL-4Rα$^{-/lox}$ mice. Lck^{cre}-negative IL-4Rα$^{-/lox}$ littermate BALB/c mice, which express similar levels of IL-4Rα as wild-type BALB/c mice, were used as controls to iLck^{cre}IL-4Rα$^{-/lox}$ mice, herein referred to as wild-type (WT) littermate control.

Commercially obtained Golden Syrian hamsters (*Mesocricetus auratus*) were used for maintenance of *L. donovani* (Harlan Olac, Bicester, UK) and *L. donovani* strain MHOM/ET/67:LV82 was used in these studies (38). Mice, age and sex-matched, were coded and randomized before injection to avoid bias. The mice were infected (day 0) by intravenous injection into the tail vein without using an anesthetic with 1–2 $\times$ 10^7 *L. donovani* amastigotes (16). Depending on the experiment, mice were killed (n = 4/5 per group) at days 14 or 15, 30, and 56 or 60 post-infection (p.i.).

In vivo Studies

Liver, spleen, and bone marrow impression smears from each mouse were prepared on a glass microscope slide at sacrifice. The slides were fixed in methanol for 2 min, stained with a 10% Giemsa solution (BDH, VWR International Ltd, UK) for 20 min, washed in tap water and allowed to dry. The number of parasites /1,000 host nuclei for each sample was determined at ×1,000 magnification. Blood was collected from each mouse at sacrifice and left to clot. The resulting serum was harvested, stored at −20°C, and then used in assays to determine specific antibody titers and cytokine levels.

Spleen Cell Proliferation Assays

Mouse spleens were removed aseptically at sacrifice, and processed as described previously (16). Splenocytes, seeded at 5 $\times$ 10^5 cells/well, were incubated at 37°C and 5% CO_2/95% air for 72 h with medium alone (unstimulated controls), 25 μg/ml *L. donovani* promastigote soluble antigen (SAG) or 10 μg/ml ConA (Sigma-Aldrich, Poole, UK). The plates were then stored at −20°C and cell supernatants were used in cytokine/nitrite assays.

Cytokine Production

Enzyme-linked immunosorbent assays (ELISA) was used to determine IFN-γ levels in splenocytes supernatants from lymphocyte proliferation assays and serum samples using the method described (19). A volume of 50 μl/well of 1 μg/ml w/v of the appropriate purified anti-mouse capture antibody [IL-10 JES5-2AS and IFNγ R4-6A2 (PharMingen, supplied by Insight Biotechnology, Wembley, UK) or IL-13 38213 (R&D Systems Europe Ltd, Abingdon, UK)] and 100 μl/well of the appropriate biotinylated rat anti-mouse monoclonal antibody at 2 μg/ml [IL-10 SXC-1 and IFNγ XMG1-2 (PharMingen, supplied by Insight Biotechnology, Wembley, UK) or IL-13 (R&D Systems Europe Ltd, Abingdon, UK)] was used in assays. The absorbance of the samples was read at 405 nm using a SOFTmax Pro (Molecular devices, California, USA) and cytokine concentrations (ng/ml) in samples was determined by linear regression using the standard values. In all cases the correlation coefficient was 0.970 or better.

Specific Antibody Titers

Specific IgG1 and IgG2a titers were determined for serum samples using the method described previously (16). Plates were coated with 100 μl/well of 10 μg/ml *L. donovani* SAG and probed using anti-mouse IgG1 (1:20,00 dilution) or IgG2a (1:10,000 dilution) HRP conjugates (Southern Biotechnology, supplied by Cambridge BioScience Ltd, Cambridge, UK). The absorbance of samples was determined at 450 nm using a SOFTmax Pro (Molecular devices, California, USA) and end point titers, defined as the last dilution to give an absorbance above background levels, was determined.

Determination of IgE Production

A 96 well microtiter plate (Greiner Bio-One, Germany) was coated with 50 μl/well of 1 μg/ml IgE purified anti-mouse capture antibody (clone R35-72 obtained from PharMingen and supplied by Insight Biotechnology, Wembley, UK) diluted in PBS pH 9.0 and incubated at 4 °C overnight. The plate was washed three times with PBS supplemented with 0.05% v/v Tween 20 (Sigma-Aldrich, Poole, UK), dried and blocked with 10% v/v FCS/PBS. Following addition of 200 μl/well the plate was incubated at 37°C for 1 h. The plate was washed 3 times, dried and serum samples diluted at 1/50 were serially diluted in 10% v/v FCS/PBS along the plate. The plate was incubated at 37°C for 2 h. Following incubation, the plate was washed 4 times and 100 μl/well of a biotinylated rat anti-mouse IgE monoclonal antibody (clone R35-118 obtained from PharMingen and supplied by Insight Biotechnology, Wembley, UK) diluted 1/5,000 in 10% v/v FCS/PBS was added to the plate. The plate was then incubated at 37°C for 1 h. The plate was washed 5 times and 100 μl/well of streptavidin alkaline phosphatase (obtained from PharMingen and supplied by Insight Biotechnology, Wembley, UK) diluted at 1/2,000 in 10% v/v FCS/PBS was added. The plate was incubated at 37°C for 45 min. The plate was washed 6 times and 100 μl/well of substrate (1 mg/ml p-nitrophenylphosphate (Sigma-Aldrich, Poole, UK) in 0.1 M glycine buffer, pH 10.4) was added. The plate was incubated in the dark at 37°C until an appropriate yellow color developed. The absorbance of the samples at 405 nm was determined using a SOFTmax Pro (Molecular devices, California, USA) and the endpoint dilution determined as described above.

Histology

Sections of liver were removed at sacrifice, fixed in neutral buffered formalin, and then processed for staining with Haematoxylin and Eosin (Fisher Scientific, Loughborough, UK). Granulomas were scored on their level of maturity based on the following criteria; infected Kupffer cells (parasitized macrophages), immature (developing granuloma consisting of CD4$^+$ and CD8$^+$ T cells and monocytes surrounding infected Kupffer cells), mature (more developed than immature) or sterile (parasite free granuloma) (39).

Flow Cytometry

The percentage of CD4$^+$IFNγ$^+$ and CD8$^+$IFNγ$^+$ cells present in the spleen of mice was determined by flow cytometry. Single cell suspensions were prepared from the spleens of uninfected and infected mice in complete medium [RPMI 1640 (Lonza, Belgium) supplemented with 1% v/v of 2 mM L-glutamine solution and 1% v/v of 100 IU/ml Penicillin-100 μg/ml Streptomycin (PAA Laboratories, GmbH, Austria), and 10% v/v FCS (Sigma Aldrich, Poole, UK)]. Cell suspensions were pelleted by centrifugation at 1,000 rpm, 4°C for 5 min and the pellets were resuspended in 3 ml of erythrocyte lysing solution (0.007 M ammonium chloride, 0.085 M Tris, pH 7.2). Cell suspensions were incubated at 37°C for 5 min, washed three times in complete medium, and then resuspended at a concentration of 1×10^6/ml. The cells were incubated for 4 h with 2 μl/ml Brefeldin A (BD Biosciences, UK), 500 ng/ml ionomycin and 50 ng/ml phorbol 12-mysristate 13 acetate (Sigma Aldrich, Poole, UK) at 37°C and 5% CO_2. The cells were then washed with FACS buffer (5% w/v Bovine serum albumin, Fraction V (Sigma Aldrich, Poole, UK), 2 mM EDTA in PBS pH 7.4) for 5 min at 500 g, and then resuspended in FACS buffer containing 1 μg/ml purified rat anti-mouse CD16/CD32 (FCγ111/11 receptor) monoclonal antibody (BD Biosciences, Oxford, UK) and incubated for 20 min. The cells were washed as before and stained with the appropriate anti-mouse antibody diluted in FACS buffer (0.5 μg/ml PerCP-labeled anti-CD4$^+$ or 1 μg/ml APC-labeled anti-CD8$^+$ or 0.5 μg/ml PerCP and 1 μg/ml APC-labeled IgG isotype controls, BD Biosciences, Oxford, UK) in the dark for 60 min at 4°C. Cells were washed three times and then incubated with a 10% v/v cell lysing solution diluted in distilled water (BD Biosciences, Oxford, UK) for 10 min in the dark. The cells were washed again and incubated for a further 10 min in the dark with a 10% v/v permeabilizing solution diluted in distilled water (BD Biosciences, Oxford UK). Following this incubation, cells were washed and resuspended in 2 μg/ml purified rat anti-mouse CD16/CD32 (FCγ111/11 receptor) monoclonal antibody (BD Biosciences, Oxford, UK) diluted in FACS buffer. After a 10 min incubation, PE-labeled anti-mouse IFNγ (final concentration 2 μg/ml) and PE-labeled IgG isotype control (final concentration 2 μg/ml, BD Biosciences, Oxford, UK) diluted in 10% v/v permeabilizing solution was added to the appropriate sample and the cells were incubated for 60 min. The cells were washed once more, and then resuspended in 400 μl of a 10% v/v cell fix solution diluted in distilled water (BD Biosciences, Oxford, UK). The number of positive staining cells for a specific marker was determined using a FACSCanto^{TM} (BD Biosciences, Oxford, UK). Color compensation using BD^{TM} Compbeads and the antibodies used to stain cells was carried out before cell data was collected based on forward and side scatter using FACsDiva^{TM} software.

Statistical Analysis of Data

Downstream data analysis was performed blind to treatment group and experiments were repeated at least twice when significant differences between treatments were obtained. Parasite burden from *in vivo* experiments were analyzed using a one-way ANOVA using $\log_{10}$ transformed data, followed by a Fisher's PLSD test to analyze differences between treatments using Statview® version 5.0.1 software package. Significant

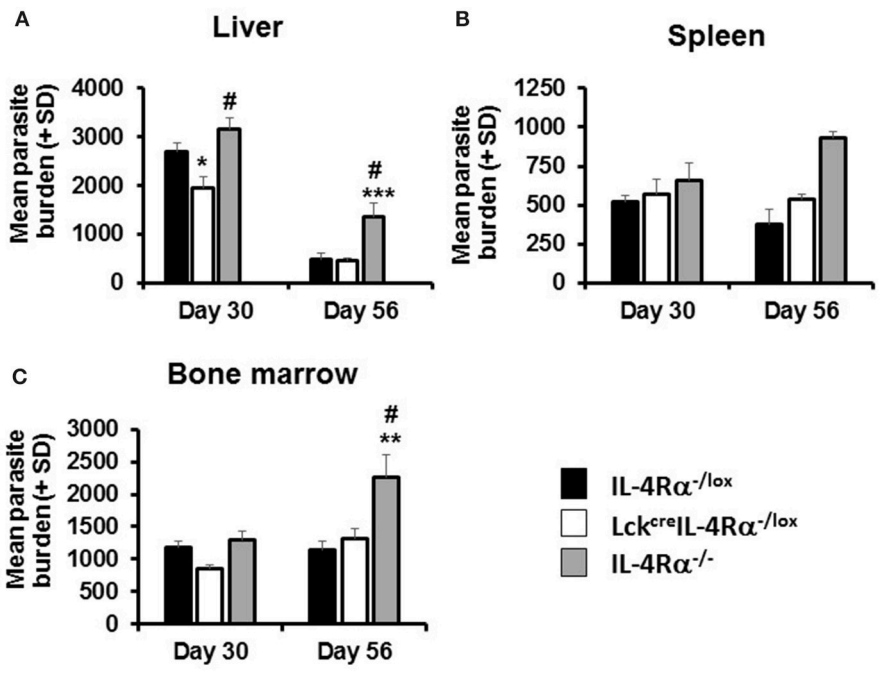

FIGURE 1 | Parasite burdens in wild-type (IL-4Rα$^{-/lox}$), CD4 T cell-specific IL-4Rα deficient (Lck^{cre}IL-4Rα$^{-/lox}$), and IL-4Rα$^{-/-}$ BALB/c mice were determined at days 30 and 56 post-infection with 1–2 × 10^7 *L. donovani* amastigotes in the liver **(A)**, spleen **(B)**, and bone marrow **(C)** by LDU. Representative data from one of three experiments performed ($n = 5$/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to WT control (IL-4Rα$^{-/lox}$), #$p < 0.05$ Lck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$.

differences between treatments for cytokine and flow cytometry data were identified using a Kruskal Wallis test followed by a Tukey test. Granuloma maturation data was analyzed using a χ^2 test and the mean % granulomas in the Kupffer cell, immature, mature and sterile categories. Data was analyzed using Minitab Express^{TM} version 1.51 software package and a $p < 0.05$ was considered significant.

RESULTS

A Protective Role for IL-4 During *L. donovani* Infection Involves Cell Targets Other Than CD4^{+} T Cells

CD4^{+} T cell-specific IL-4Rα deficient (Lck^{cre}IL-4Rα$^{-/lox}$) and wild-type littermate control (IL-4Rα$^{-/lox}$) and global IL-4Rα$^{-/-}$ BALB/c mice were infected with *L. donovani* on day 0 and parasite burdens in the spleen, liver, and bone marrow were determined, on day 14/15, 30, and 56/60 p.i. All three mouse strains had similar parasite burdens in the spleen, liver, and bone marrow at early times p.i. (day 14 or 15; **Table S1**), indicating that the inability to signal through IL-4Rα on CD4^{+} T cells did not interfere with establishment and early control of infection in the spleen, liver, and bone marrow.

At day 30 p.i., CD4^{+} T cell IL-4Rα deficient mice and littermate controls had comparable parasite burdens in the spleen and bone marrow. However, CD4^{+} T cell IL-4Rα deficient mice had significantly lower liver parasite burdens than WT littermate control and global IL-4Rα$^{-/-}$ BALB/c mice (**Figure 1**) at this time. This effect was transient however, and by day 56 parasite burdens in all three sites was comparable between WT and CD4^{+} T cell-specific IL-4Rα deficient mice. In contrast, and as expected (15), global IL-4Rα$^{-/-}$ mice were more susceptible to *L. donovani* infection and this was exhibited by significantly higher parasite burden at later time points in all target tissues (**Figure 1**). Thus, selective deficiency of IL-4Rα expression in CD4^{+} T cells had a temporary protective effect, which was only expressed in the liver, but did not alter the overall susceptibility to VL.

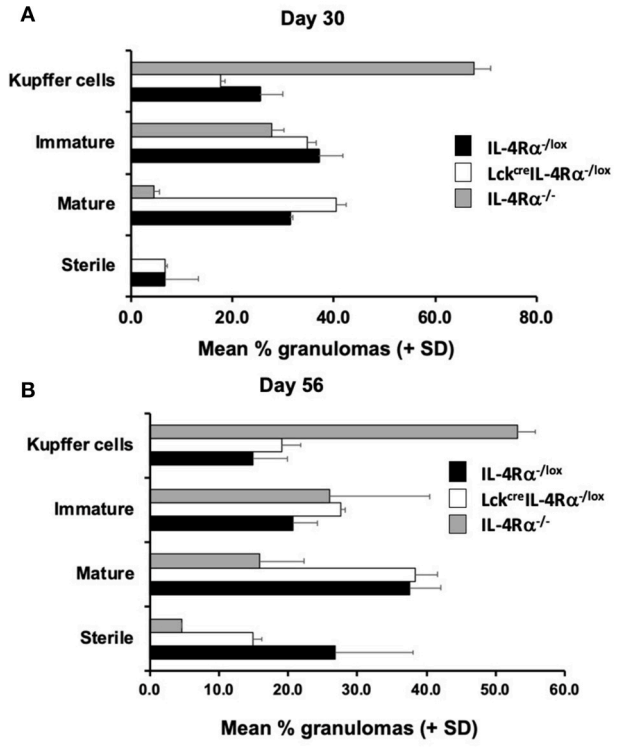

FIGURE 2 | CD4^{+} T cell specific IL-4Rα$^{-/lox}$ (Lck^{cre}IL-4Rα$^{-/lox}$), wild-type control (IL-4Rα$^{-/lox}$), and global IL-4Rα$^{-/-}$ BALB/c mice were infected with *L. donovani* on day 0 post-infection and sacrificed at days 30 **(A)** and 56 **(B)** post-infection. At each time-point, sections of liver were removed, processed, and stained with haematoxylin and eosin to enable scoring of hepatic liver granulomas. Representative data from one of two experiments performed (n = 5/group). On day 30 **(A)** the distribution was significantly different for control and global IL-4Rα$^{-/-}$ mice ($\chi^2 = 44$; $p < 0.00001$) and CD4^{+} T cell specific IL-4Rα$^{-/lox}$ (Lck^{cre}IL-4Rα$^{-/lox}$) and global IL-4Rα$^{-/-}$ mice ($\chi^2 = 63$; $p < 0.00001$). On day 56 **(B)** the distribution was significantly different for control and global IL-4Rα$^{-/-}$ mice ($\chi^2 = 46$; $p < 0.00001$) and CD4^{+} T cell specific IL-4Rα$^{-/lox}$ (Lck^{cre}IL-4Rα$^{-/lox}$) and global IL-4Rα$^{-/-}$ mice ($\chi^2 = 30$; $p < 0.00001$).

IL-4 Signaling Via CD4^{+} T Cells Is Not a Requirement for Effective Hepatic Granuloma Formation Following *L. donovani* Infection

Granuloma formation in livers of *L. donovani* infected mice was assessed at days 15, 30, and 56 post-infection in CD4^{+} T cell specific IL-4Rα deficient (Lck^{cre}IL-4Rα$^{-/lox}$), wild-type littermate control (IL-4Rα$^{-/lox}$) and global IL-4Rα$^{-/-}$ BALB/c mice. At day 15 post-infection, although granuloma development was evident, the granulomas were at an immature stage and, as such, no significant differences between groups were observed (**Table S2**). At day 30 post-infection, however, CD4^{+} T cell-specific IL-4Rα deficient mice and wild-type controls showed evidence of granuloma maturation, as defined by an increased number of mature and sterile granulomas, compared with global IL-4Rα$^{-/-}$ mice (**Figure 2A**). Similarly, at day 56, the frequency of mature and sterile granulomas had increased and was similar in CD4^{+} T cell-specific IL-4Rα deficient mice and wild-type control mice, reflecting a similar ability to control liver parasite burdens by this time point (**Figure 2B**). Granuloma maturation was significantly retarded in global IL-4Rα$^{-/-}$ but not CD4 T cell-specific compared with WT mice at both time points ($p < 0.00001$). Representative photomicrographs show granuloma formation in each group at days 15 (**Figure S1**), 30 (**Figures 3A–C**) and day 56 (**Figures 3D–F**) post-infection.

Th2/type 2 Immune Responses Are Unchanged in CD4 T Cell-Specific IL-4Ra Deficient Mice in Response to *L. donovani*-induced VL

An inability of global IL-4Rα$^{-/-}$ mice to control parasite burdens or develop a mature and effective granulomatous response has previously been associated with a down-regulation in serum IFN-γ production (15). In the present study we therefore measured production of IFN-γ by antigen-stimulated spleen

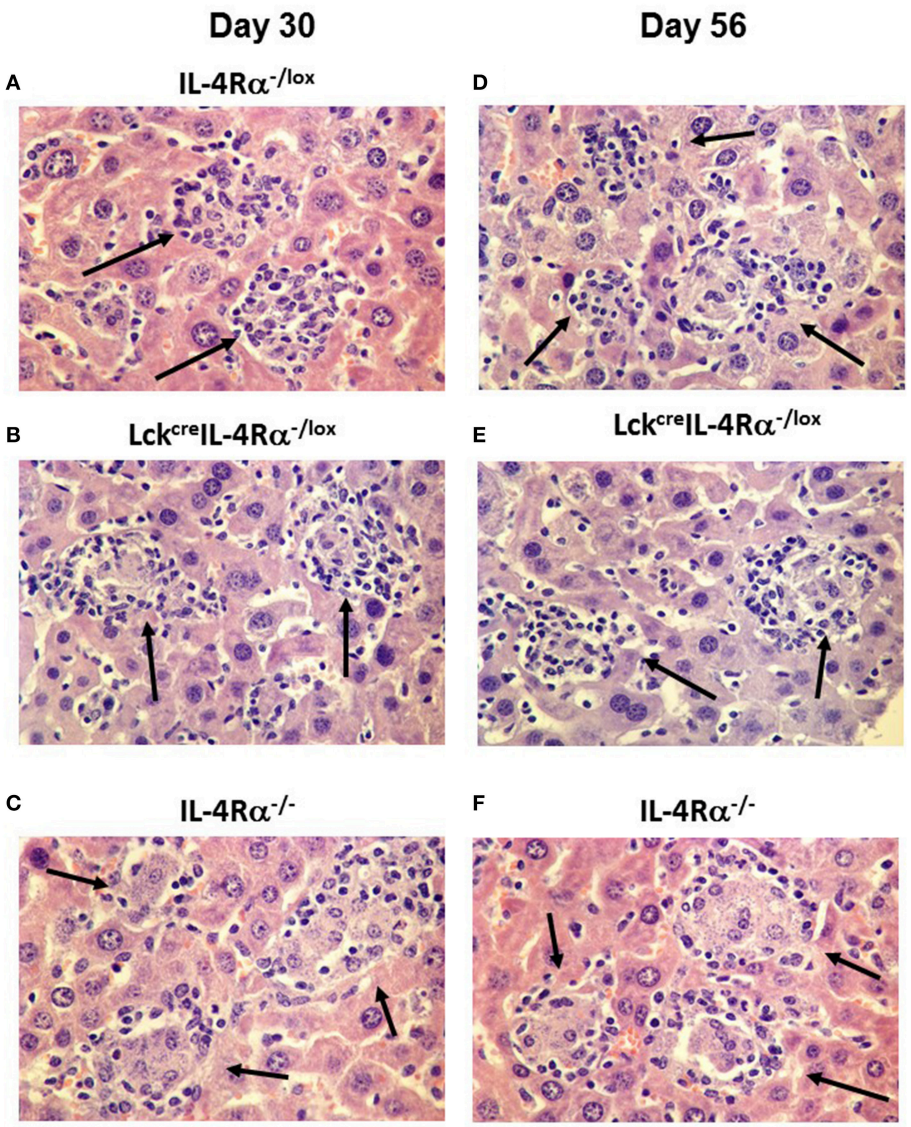

FIGURE 3 | Representative photomicrographs of the hepatic granuloma response at days 30 and 56 in *L. donovani* infected wild-type (IL-4Rα$^{-/lox}$) **(A,D)**, CD4^{+} T cell specific IL-4Rα$^{-/lox}$ (Lck^{cre}IL-4Rα$^{-/lox}$) **(B,E)** and global IL-4Rα$^{-/-}$ mice **(C,F)**. Mature granulomas (arrows) in IL-4Rα$^{-/lox}$ mice and Lck^{cre}IL-4Rα$^{-/lox}$ mice are shown in day 30 whilst global IL-4Rα$^{-/-}$ mice show abrogated granuloma development characterized by immature granulomas and amastigotes within the cytoplasm of infected Kupffer cells (arrows). At day 56 post-infection, IL-4Rα$^{-/lox}$ mice and Lck^{cre}IL-4Rα$^{-/lox}$ mice show sterile granulomas (arrows) whilst heavily parasitized immature granulomas remain in the livers of global IL-4Rα$^{-/-}$ mice (arrows). Magnification 400×.

cells and the frequency of CD4^{+}IFN-γ^{+} and CD8^{+}IFN-γ^{+} splenocytes of each mouse group after stimulation with PMA and ionomycin, as a measure of commitment to cytokine production during infection. On day 30 post-infection, we found no significant difference in the amount of IFN-γ [ng/ml] produced by antigen-stimulated splenocytes from any group of mice, WT controls (0.207 ± 0.030), CD4^{+} T cell specific IL-4Rα deficient mice (0.331 ± 0.049), global IL-4Rα$^{-/-}$ mice (0.482 ± 0.076). However, using flow cytometry to examine intracellular cytokine production at this time revealed that the percentages of CD4^{+}IFN-γ^{+} splenocytes (**Figure 4A**) and CD8^{+}/IFN-γ^{+} splenocytes (**Figure 4B**) in global IL-4Rα$^{-/-}$ mice were significantly lower (CD4^{+}IFN-γ^{+}, $p < 0.05$; CD8^{+}IFN-γ^{+}, $p < 0.02$) than observed in WT controls while the frequency of CD8^{+}/IFN-γ^{+} splenocytes in global IL-4Rα$^{-/-}$ mice was also reduced compared with CD4^{+} T cell specific IL-4Rα$^{-/-}$ mice ($p < 0.01$, **Figure 4B**). This pattern was repeated on day 60 post-infection, (e.g., $p < 0.0001$ for global IL-4Rα$^{-/-}$ mice vs. CD4^{+} T cell-specific IL-4Rα-deficient mice). There was no significant difference measured in the frequency of CD4^{+}IFN-γ^{+} and CD8^{+}IFN-γ^{+} cells between CD4^{+} T cell specific IL-4Rα deficient mice and wild-type controls at all time

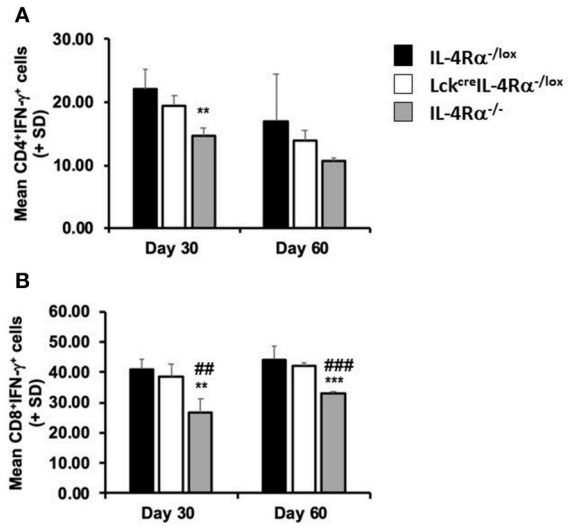

FIGURE 4 | CD4$^+$ T cell specific IL-4Rα deficient (Lck^{cre}IL-4Rα$^{-/lox}$), wild-type control (IL-4Rα$^{-/lox}$) and global IL-4Rα$^{-/-}$ mice were sacrificed at days 30 and 60 post-infection. At each time-point, splenocytes were removed, stimulated with ionomycin and PMA in the presence of brefeldin A, and the percentage of IFN-γ secreting CD4$^+$ **(A)** and CD8$^+$ **(B)** cells were measured by flow cytometry. Representative data from one of two experiments performed. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to WT control (IL-4Rα$^{-/lox}$), $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ Lck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$.

points. Together, these data indicate that IFN-γ-responses in Lck^{cre}IL-4Rα$^{-/lox}$ mice developed fully in the absence of IL-4Rα$^+$ signaling on CD4$^+$ T cells when compared with wild-type mice over the course of infection. Moreover, enhanced susceptibility to *L. donovani* infection in global IL-4Rα$^{-/-}$ mice is associated with reduced IFN-γ secretion by CD4$^+$ and CD8$^+$ T cells.

IL-10 levels are associated with susceptibility to *L. donovani* infection (14), thus, IL-10 levels were measured in cell supernatants of antigen-stimulated spleen cells and in the serum from CD4$^+$ T cell specific IL-4Rα deficient mice, wild-type control mice and global IL-4Rα$^{-/-}$ mice at days 15, 30, and 56 post-infection. There was no significant difference in the amount of IL-10 [ng/ml] produced by antigen-stimulated cells from any group of mice at day 30, WT controls (0.281 ± 0.075), CD4$^+$ T cell specific IL-4Rα deficient mice (0.231 ± 0.026), and global IL-4Rα$^{-/-}$ mice (0.230 ± 0.013). In addition, while similar levels of IL-10 were present in the serum of all three groups of mice at day 15 p.i, by day 30 p.i significantly lower concentrations of IL-10 were present in the serum of wild-type control ($p < 0.01$) and CD4$^+$ T cell specific IL-4Rα deficient mice ($p < 0.01$) compared with global IL-4Rα$^{-/-}$ mice (**Figure 5**). This pattern was repeated on day 56 post-infection. This data suggests that the relative resistance observed in CD4$^+$ T cell specific IL-4Rα deficient and littermate control mice, in comparison to highly susceptible global IL-4Rα$^{-/-}$ mice, is associated with comparatively limited IL-10 production.

CD4$^+$ T Cell Specific IL-4Rα-deficient Mice Develop *L. donovani*-specific IgG1 and IgE Antibody Responses Similar to WT Control Mice

L. donovani-specific IgG1 and IgG2a levels and total serum IgE levels were measured in CD4$^+$ T cell specific IL-4Rα$^{-/-}$ mice, wild-type control and global IL-4Rα$^{-/-}$ mice at days 15, 30, and 56 post-infection. There was no difference in specific IgG1 or IgG2a in the three groups of mice at day 14 post-infection (**Figures 6A,B**). However at day 30 p.i., global IL-4Rα$^{-/-}$ produced significantly lower levels ($p < 0.0.01$) of antigen-specific IgG1 but significantly higher IgG2a levels compared with CD4$^+$ T cell specific IL-4Rα deficient mice and wild-type controls. In contrast, IgG1 and IgG2a titers were similar in CD4$^+$ T cell specific IL-4Rα deficient mice and wild-type controls (**Figures 6A,B**). On day 60 p.i., both global and CD4$^+$ T cell specific IL-4Rα$^-$deficient mice had significantly lower IgG1 titers compared with WT controls ($p < 0.05$). IgG2a titers were similar in all three groups of mice (**Figures 6A,B**). Comparison of the ratio of IgG2a:IgG1 showed that global IL-4Rα$^{-/-}$ mice had a higher ratio at days 30 ($p < 0.01$) and 56 ($p < 0.001$) p.i. compared with WT and CD4$^+$ T cell specific IL-4Rα deficient mice (**Figure 6C**). Both CD4$^+$ T cell specific IL-4Rα deficient mice and wild-type control mice produced comparable amounts of IgE on days 30 and 56 post-infection (**Figure 6D**). In contrast, no serum IgE was detected for global IL-4Rα$^{-/-}$ mice any day post-infection (**Figure 6D**). Based on this antibody data, it can be concluded that the inability to signal through the IL-4Rα on CD4$^+$ T cells did not prevent a specific type 2 antibody response whereas the inability to class switch in the global IL-4Rα$^{-/-}$ mice resulted in a reduced type 2-antibody response.

Pan T Cell-Specific IL-4Rα Deficient Mice Remain Comparatively Resistant to VL Compared With Mice Globally Deficient for IL-4/IL-13 Signaling

As CD4$^+$ T cell-specific IL-4Rα-deficient mice remain positive for IL-4Rα expression on CD8 T cells, we could not rule out the possibility that IL-4Rα$^+$ CD8$^+$ T cells in CD4$^+$ T cell specific IL-4Rα$^-$ deficient mice could have contributed to infection control, especially since CD8$^+$ T cells have been implicated in protective immunity to VL (15, 30). Thus, we sought to investigate this further by using pan T cell specific IL-4Rα-deficient mice (iLck^{cre}IL-4Rα$^{-/lox}$) which lack IL-4Rα signaling on CD4 T cells as well as CD8 and Foxp3 regulatory T cells (33). iLck^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$ littermate control and global IL-4Rα$^{-/-}$ mice were infected with *L. donovani* and parasite burdens analyzed at day 30 p.i. in the spleen, liver, and bone-marrow. All groups of animals exhibited similar parasite numbers in the spleen and bone-marrow at day 30 p.i. However, pan T cell IL-4Rα$^{-/lox}$ exhibited disease-control in the liver compared with WT littermate controls ($p < 0.01$) and global IL-4Rα$^{-/-}$ mice ($p < 0.01$, **Table 1**), similar to but more pronounced than that seen in CD4$^+$ T cell-specific IL-4Rα-deficient mice (**Figure 1A**).

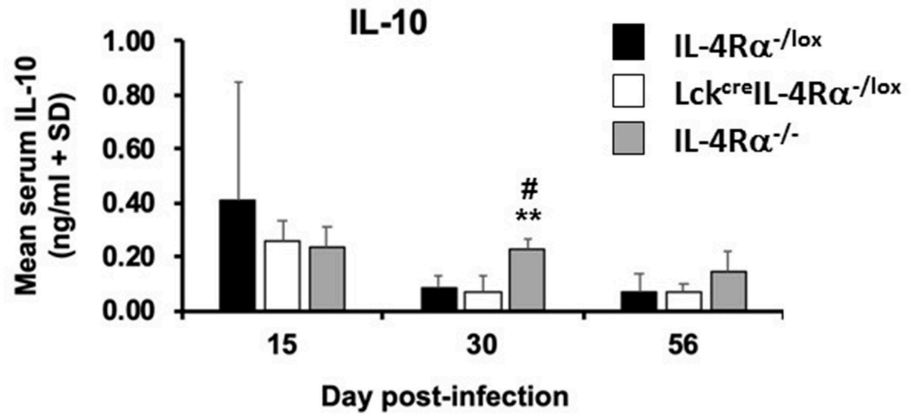

FIGURE 5 | Serum IL-10 levels in wild-type control mice (IL-4Rα$^{-/lox}$), CD4^{+} T cell specific IL-4Rα deficient mice (Lck^{cre}IL-4Rα$^{-/lox}$) and global IL-4Rα$^{-/-}$ over the course of infection were determined by ELISA against *Leishmania donovani* soluble antigen. Representative data from one of two experiments performed (n = 5/group). $^{**}p < 0.01$ compared to WT control (IL-4Rα$^{-/lox}$), $^{\#}p < 0.05$ Lck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$.

Altogether, these data indicate that IL-4Rα signaling on T cells transiently exacerbates infection in the liver after infection with *L. donovani*. These results also reinforce the ability of IL-4/IL-13 to exhibit temporal and spatial regulation depending on cell-type and host tissue involved during infection. However, given that IL-4/IL-13 signaling globally is protective in VL, our results clearly demonstrate that the T cell is ultimately not the IL-4Rα responsive target mediating this protection.

DISCUSSION

While it is well-established that protective immunity against leishmaniasis, whether cutaneous or visceral, relies upon an IL-12-driven type 1 response and IFN-γ production, the contribution of IL-4, IL-13, and signaling via IL-4Rα to the outcome of *Leishmania* infection is very much parasite-species dependent [reviewed (21, 40)]. Numerous studies have identified detrimental roles for IL-4, IL-13, and signaling via IL-4Rα, as well as IL-10, during cutaneous infection with *L. major* and *Leishmania mexicana* complex parasites. In contrast, experimental studies using gene-deficient mice in *L. donovani* infections have indicated that the control of not only primary infection, but also successful chemotherapy and successful vaccination is IL-4, IL-13, and IL-4Rα signaling-dependent (15–17, 32). The absence of these cytokines, or the inability to signal via the IL-4Rα in BALB/c mice, results in a reduced IFN-γ response, severely limited granuloma development, enhanced IL-10 production and disease exacerbation. However, the mode of action of these cytokines continues to remain obscure as IL-4 and IL-13 are pleiotropic cytokines and many cell types are not only able to produce these cytokines but also are responsive to them via IL-4Rα (21). Consequently, the generation of spatial (cell-specific) IL-4Rα$^{-/-}$ mice has provided an invaluable resource to identify the specific role of IL-4/IL-13-responding cells in ongoing immune responses (15, 17, 19). A previous study by us using macrophage/neutrophil specific IL-4Rα-deficient BALB/c mice identified no role, whether protective or detrimental, for IL-4 or IL-13 signaling via these cells on the outcome of primary *L. donovani* infection (16). T cells are the primary source of IFN-γ production in acquired immunity against VL (30), and have been implicated as playing a major role in granuloma formation and resolution of infection in the murine model (39). Therefore, in the present study we utilized CD4^{+} T cell-specific IL-4Rα-deficient (Lck^{cre}IL-4Rα$^{-/lox}$) mice (23), and as confirmation iLck^{cre}IL-4Rα$^{-/lox}$ mice that lack IL-4Rα on both CD4^{+}and CD8^{+} T cells (33) to determine if IL-4Rα signaling via T cells plays any important role in protection. The results reveal that IL-4Rα signaling via the CD4^{+} T cell is not a requirement for successful resolution of *L. donovani* infection, but the inability to signal via IL-4Rα did have a transient protective effect (day 30) on hepatic parasite burdens compared with wild-type counterparts. This hepatic protective effect was also observed in pan-T cell specific BALB/c mice i.e., iLck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice, confirming that this early response is mediated by IL-4Rα signaling on T cells. However, later post-infection, CD4^{+} T cell specific IL-4Rα-deficient mice and wild-type controls had equivalent parasite burdens in the spleen, liver and bone marrow as well as a similar hepatic granulomatous response. This is in contrast to global IL-4Rα$^{-/-}$ mice that developed significantly higher parasite burdens and abrogated hepatic granuloma maturation compared with CD4^{+} T cell specific IL-4Rα-deficient and wild-type mice in all tissue sites. Overall these data suggest that host-protection mediated by IL-4/IL-13 globally during *L. donovani* infection is not due to IL-4 acting on CD4^{+} T cell populations.

Earlier studies on *L. donovani*, not only in mice (9), but also humans (10–12), suggested that control of infection was independent of the differential production of type 1 and type 2 cytokines and murine studies indicate that protection is related to increasing the frequency of cytokine-producing cells rather than altering the IFN-γ/IL-4 balance (13). The results presented here confirm that resistance to *L. donovani* and induction

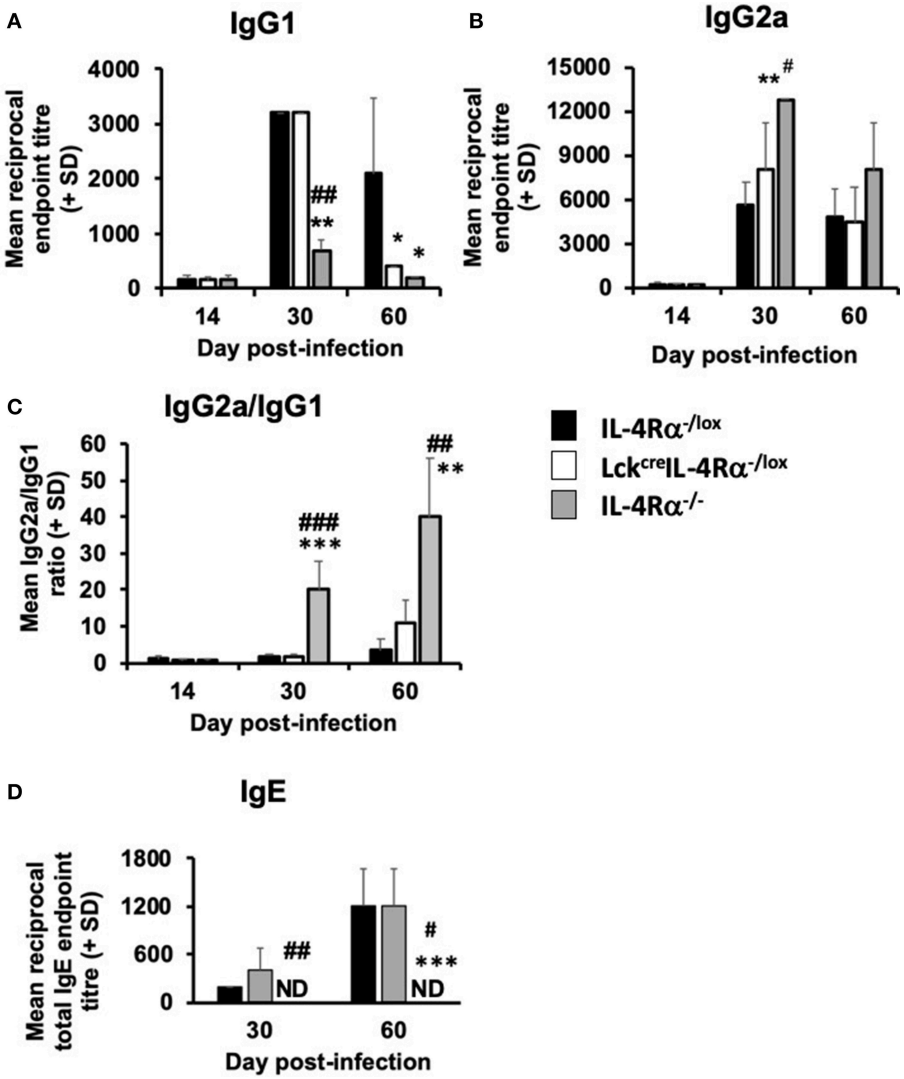

FIGURE 6 | The effect of *L. donovani* infection on the antibody response of *L. donovani* infected CD4^{+} T cell specific IL-4Rα deficient (Lck^{cre}IL-4Rα$^{-/lox}$), wild-type control (IL-4Rα$^{-/lox}$), and global IL-4Rα$^{-/-}$ mice. Antigen-specific IgG1 **(A)**, IgG2a **(B)**, the ratio of antigen-specific IgG2a/IgG1 **(C)** and total IgE serum levels are shown **(D)**. Specific antibody titers were determined using an antigen-specific ELISA and total IgE levels were also determined using a direct ELISA assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to WT control (IL-4Rα$^{-/lox}$), #$p < 0.01$, ##$p < 0.01$, ###$p < 0.001$ Lck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$. Representative data from one of two experiments performed ($n = 5$/group).

of effective granuloma production is dependent on successful generation of an IFN-γ response on both CD4^{+} and CD8^{+} T cells and while this is positively regulated by IL-4/IL-13 signaling on a global scale, it is not strictly dependent on CD4^{+} and CD8^{+} T cells signaling these cytokines. CD4^{+} T cells (26, 39) and CD8^{+} T cells (41) produce IFN-γ, which activates macrophages to produce antimicrobial reactive nitrogen and oxygen intermediates (7), and these are also important in driving granuloma maturation. The observations on comparative T cell IFN-γ expressing cell numbers from this study complement and reinforce our previous observation that global IL-4Rα$^{-/-}$ mice produce significantly lower levels of serum IFN-γ compared with wild-type mice following *L. donovani* infection, and contribute to their abrogated granuloma development (15).

In parallel, although IL-4 is important in proliferation and maintenance of CD4^{+} Th2 cells, it is not required for early production of Th2 cells (42, 43), and therefore the inability to signal via the IL-4Rα did not prevent the induction of antigen-specific Th2 cell response, shown by the similar antigen-specific IgG1 titers in *L. donovani* infected WT and CD4^{+} T cell-specific IL-4Rα-deficient (Lck^{cre}IL-4Rα$^{-/lox}$) mice. This may not be unexpected as it has been suggested that antigen-specific Th2 cells is the default position for Th cells (41). The ability to produce antigen-specific IgG1 in the absence of IL-4Rα signaling has been reported in other studies (39, 40).

TABLE 1 | Parasite burdens of WT (IL-4Rα$^{-/lox}$), pan T cell IL-4Rα-deficient (iLck^{cre}IL-4Rα$^{-/lox}$), and IL-4Rα$^{-/-}$ deficient BALB/c mice in the spleen, liver and bone marrow.

Strain	Mean parasite burden (+ SD)		
	Spleen	Liver	Bone marrow
IL-4Rα$^{-/lox}$	329 ± 87	2554 ± 334	645 ± 94
iLck^{cre}IL-4Rα$^{-/lox}$	285 ± 47	1040 ± 376**	494 ± 77
IL-4Rα$^{-/-}$	509 ± 87	2279 ± 376#	875 ± 145

*Mice (n = 5/treatment) were infected with L. donovani LV82 and parasite burdens were determined on day 30 post-infection. **p < 0.001 compared to WT control, **p < 0.01 compared to WT control, #p < 0.01 iLck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$.*

Recently, a pivotal role for IL-4 and IL-4Rα-responsive B cells in the non-healing response of BALB/c mice infected with *L. major* has been demonstrated (25). Abrogation of IL-4Rα signaling on B cells in BALB/c mice (mb1^{cre}IL-4Rα$^{-/lox}$) turned non-healer BALB/c mice into a healer phenotype concomitant with a switch from a predominately Th2 to a Th1 response. Regulatory B cells producing IL-10 have also been associated with non-healing *L. major* infection (44) although apparently not associated with IL-4 responsiveness. However, the role of B cells (as APCs or regulatory cells) and/or antibody in contributing to susceptibility to *Leishmania* infection appears to depend in large part upon the parasite species examined but also probably to a significant extent upon the host. B cells have been shown to play a role in VL, thus B cell-deficient C57BL/6 mice infected with *L. donovani* (45) and BALB/c mice infected with *L. infantum* (46) are relatively resistant to infection. Susceptibility to infection in B cell deficient C57BL/6 mice, unlike BALB/c mice is not dependent on antibody production, although antibodies are associated with protection in infected C57BL/6 mice as they prevent excessive pathology. Marginal zone B cells regulate antigen-specific CD8^{+} T cells responses (47), indicating that B cells may be protective via their ability to present antigen. In *L. infantum*-infected BALB/c mice, neither IL-10 production by B cells nor antigen presentation by B cells is involved in disease exacerbation. However, passive transfer experiments have shown that IgM and IgG, induced by polyclonal B cell activation during infection, promote parasite growth. It is possible that the role of B cells differs in the two mouse strains and is related to their inherent "cure" phenotype (C57BL/6) and "non-cure" phenotype in *L. donovani* (48). B cells have been observed to have cognate interactions with T cells in hepatic granulomas in BALB/c mice (28) and problems with antigen presentation to CD8^{+} T cells have been reported in *L. donovani* (30). There were clear differences in antibody responses between global IL-4Rα$^{-/-}$ mice and the other two strains, with differences in total IgE and antigen-specific IgG1 being the most marked. Production of these antibody classes by B cells is known to be controlled by IL-4/IL-13 and requires IL-4Rα signaling (49). The fact that CD4^{+} T cell specific IL-4Rα$^{-/-}$ mice can still produce IgE indicates that a comparative examination of *L. donovani* disease phenotypes in B cell specific IL-4Rα$^{-/lox}$ BALB/ c mice would be worthwhile.

Both human (12) and murine studies (14, 50) indicate that IL-10, rather than IL-4/IL-13 is the major immunosuppressive cytokine in VL. The source of this IL-10 is not traditional Th2 cells (13) but perhaps a regulatory T cell population (51), and different types of regulatory T cells have been identified in *L. donovani*, including CD4^{+}CD25^{-}FoxP3^{-} T cells in humans (52), and CD4^{+}CD25^{-}FoxP3^{+} in mice (53) and humans (54). In addition, numerous non-T cell populations, including DCs (55) and NK cells (56) have been implicated as playing regulatory roles during VL. Thus, *L. donovani* infected IL-10 gene deficient mice demonstrated increased control over visceral infection and enhanced granuloma formation, whilst IL-10 transgenic mice developed a severe progressive disease (50, 57) clearly demonstrating that the presence of IL-10 can prevent efficient granuloma development (50). Indeed, in the present study, global IL-4Rα$^{-/-}$ mice had impaired hepatic granuloma development alongside elevated serum IL-10 levels compared with wild-type control mice and CD4^{+} T cell specific IL-4Rα$^{-/-}$ mice. Interestingly it has previously been shown that IL-4 inhibits IL-10 to promote IL-12 production in DCs in the presence of CpG or LPS (58), or *Cryptococcus neoformans* (59), while IL-13 can induce DC IL-12 production *in vitro* when used to prime DC prior to LPS stimulation (16). IL-4 treatment of BALB/c mice pre-T cell priming has previously been demonstrated to instruct DC to produce IL-12 and facilitate a protective Th1 response against *L. major* (60). In addition, deletion of IL-4Rα on DCs renders BALB/c mice hypersusceptible to *L. major* (22). DC IL-12 production in the early phase of *L. donovani* infection (61–64), in particular, has been identified as directing immune responses influencing granuloma formation during infection. Consequently, these observations, collectively, would clearly point to DCs as very probable targets of the IL-4/IL-13 induced protective response identified from our studies on IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-4Rα$^{-/-}$ mice.

CONCLUSION

A significant number of studies from our laboratories have identified protective roles for IL-4, IL-13 and IL-4Rα signaling, not merely during primary infection with *L. donovani* infection (15–17), but also for effective sodium stibogluconate chemotherapy (16, 19). In addition, there is a requirement on IL-4/IL-13 to instruct a protective type 1 response mediated by CD8^{+} T cells in HASPB-1 vaccination against *L. donovani* (65). Given the pleiotropic nature of the IL-4Rα, these IL-4Rα-responsive cells could of course be different populations in different tissue sites and could vary for different type of immune responses e.g., primary infection, response to chemotherapy or successful vaccination. In lieu of this dynamic regulation, as yet our studies have failed to identify the IL-4Rα-expressing cells mediating protection during VL. So far we have ruled out a protective role in VL for IL-4Rα responsiveness on neutrophils and macrophages in primary infection and chemotherapy, and herein T cells during primary infection. Interestingly, the primary source of IL-4 in HASPB-1 vaccination studies was defined as a CD11b^{+}CD11c^{lo} phagocyte (65) and as this

source is clearly not macrophages/neutrophils (16), alternate phagocytes expressing CD11b may regulate host immunity to VL. A candidate phagocyte for this role could be DCs given the "IL-4 instruction theory," defined as the ability of early IL-4, signaling via the IL-4Rα on DCs, to instruct early IL-12 production to promote Th1-responses (22, 60). We are currently investigating the exact mechanism to explain how this works as well as generating a variety of additional IL-4Rα cell specific knockouts to allow further dissection of the immunological mechanisms responsible for IL-4Rα mediated protection against VL. Ultimately identifying the IL-4Rα responsive host cells mediating protection will have significant implications in the rational design of new host-directed therapeutic strategies.

DATA AVAILABILITY

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

AUTHOR CONTRIBUTIONS

JA, FB, and PK: conceptualization. EM, KC, TM, and RH: methodology and investigation. EM, KC, JA, and RH: writing, review, and editing of manuscript. FB: cell-specific gene-deficient mice.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01957/full#supplementary-material

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MINI REVIEW
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Parasite-Produced MIF Cytokine: Role in Immune Evasion, Invasion, and Pathogenesis

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Protozoan parasites represent a major threat to health and contribute significantly to morbidity and mortality worldwide, especially in developing countries. This is further compounded by lack of effective vaccines, drug resistance and toxicity associated with current therapies. Multiple protozoans, including *Plasmodium*, *Entamoeba*, *Toxoplasma*, and *Leishmania* produce homologs of the cytokine MIF. These parasite MIF homologs are capable of altering the host immune response during infection, and play a role in immune evasion, invasion and pathogenesis. This minireview outlines well-established and emerging literature on the role of parasite MIF homologs in disease, and their potential as targets for therapeutic and preventive interventions.

Keywords: MIF, cytokine, protozoan parasites, host-parasite interaction, immune evasion, immunopathology, immunotherapeutic target

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INTRODUCTION

Protozoan parasites cause more than one million deaths annually. For example, *Plasmodium falciparum*, a protozoan parasite responsible for most human malaria, accounted for an estimated 200 million malaria cases and roughly 500,000 malaria deaths in 2015 (1, 2). The protozoan parasite *Leishmania* causes an estimated 50,000 deaths per annum through visceral leishmaniasis (3). *Entamoeba histolytica* is a protozoan parasite that causes colitis (inflammatory diarrhea). Millions of people are infected with *E. histolytica*, making amebic colitis a leading cause of severe diarrhea, estimated to kill more than 50,000–100,000 people each year (4–6). The protozoan parasite *Toxoplasma gondii*, which affects up to a third of the world's population, is adapted to survive and abide chronically in its host (7). The threats posed by protozoan parasites are further compounded by lack of any effective parasite vaccine, emerging drug resistance, drug toxicity, poor efficacy, and limited antimicrobial options (5, 8, 9). Therefore, identifying novel targets for therapeutic intervention and vaccine prevention is urgently needed. Pathogenic protozoans produce virulence factors that enable immune response evasion and host invasion which promote their transmission and ability to cause human disease (10). Targeting these virulence factors required to cause host damage and disease might successfully treat and prevent these infectious diseases.

The pathogenesis of protozoan diseases is highly variable, and is often influenced by individual life cycles and immunologic consequences of infection. The complicated life cycle of *Plasmodium* begins when an infected female anopheles mosquito injects sporozoites into the bloodstream of a human during a blood meal, which travel to the liver, before emerging to release merozoites into the bloodstream. These merozoites invade and multiply within erythrocytes to rupture, releasing more merozoites, and continually perpetuating invasion by the parasite. *Plasmodium* promotes

its survival by avoiding excessive exposure to the immune system by infecting hepatocytes and erythrocytes. Clinical symptoms are associated with the rupture of infected erythrocytes and the release of malarial toxins, and include fever, severe hemolytic anemia and other systemic features. Merozoites also develop into sexual forms known as gametocytes, which are ingested during mosquito bites to continue the life cycle (11). Similarly, *Leishmania* is also a vector-borne protozoan parasite, that is transmitted when *Leishmania* promastigotes are inoculated into the subdermis of the skin by the bite of an infected female phlebotomine sand fly. *Leishmania* is rapidly phagocytized by neutrophils. Promastigotes within dead infected neutrophils are taken up by host macrophages, morphing into the amastigote form. Depending on the species, amastigotes replicate within the macrophage locally to form disfiguring skin ulcers (cutaneous leishmaniasis) or disseminate to the bone marrow, liver, and spleen (visceral leishmaniasis) which is fatal if untreated (12–15).

In contrast to these vector-borne infections, the transmission of the highly prevalent protozoa, *Toxoplasma gondii,* is fecal-oral, through the ingestion of the oocyst from material contaminated with feline feces or undercooked meat infected with tissue cysts. Following intestinal infection, tachyzoites form, and then disseminate to other tissues in the body including the brain, eye, muscle, liver, and placenta. Like *Leishmania*, Toxoplasma is able to infect phagocytes, which facilitates successful infection. Symptoms of primary infection include fever, adenopathy, headache, and myalgia. The stimulation of a robust immune response controls the acute infection, driving the parasite into a chronic, asymptomatic stage allowing *Toxoplasma* to survive as bradyzoites in cyst forms within multiple tissues capable of later reactivation (7). Infection with *E. histolytica* also begins with the ingestion of fecally contaminated food or water, but has a relatively simpler life cycle. *E. histolytica* exists as either infective cysts which are ingested or transforms into invasive trophozoites that penetrate the mucus layer of the large intestine to cause colitis leading to diarrhea, dysentery, and colonic ulceration. The trophozoites can also on occasion disseminate to cause extra-intestinal disease, with a particular predilection for the liver leading to amebic liver abscess (16). Thus, in order to complete their life cycle, all of these protozoa must be able to invade and pass from host to host while avoiding clearance by the immune response. In this minireview, we describe how protozoa secrete a specific protein macrophage migration inhibitory factor to accomplish this task.

MACROPHAGE MIGRATION INHIBITORY FACTOR

Macrophage migration inhibitory factor (MIF) was one of the first cytokines to be discovered over 50 years ago (17, 18). Since then, a significant amount of information has been accumulated regarding the role of MIF in normal physiology and pathology. MIF is a well-studied pleiotropic inflammatory protein, expressed by a variety of cells, and is a critical upstream mediator of innate immunity. While MIF's exact molecular mechanism is not fully understood, partial pathways of MIF signaling have been established. For example, secreted MIF binds to its receptor, CD74, on immune cells, activates the ERK1/2 and PI3K/Akt pathways, and modulates expression of various cytokines, e.g., TNF-α, IL-6, IL-8, and IL-12 (19). MIF may also bind to CXCR2 and CXCR4, which may be responsible for its chemotactic properties. In addition, MIF stimulates the production of matrix metalloproteinases (20). Therefore, it is not surprising that MIF plays an important role in immunity and that excess MIF expression has been linked to exaggerated inflammation and immunopathology in diseases such as rheumatoid arthritis, and inflammatory bowel disease (19, 21, 22).

The proinflammatory properties of MIF also make it a crucial mediator in the immune response against a wide variety of pathogens including parasites (23). In protozoan infection, host MIF play a key role in reducing parasite burden through stimulation of both innate and adaptive immune cells. Mechanistically, host MIF can stimulate nitric oxide production by macrophages and dendritic cells, which in turn eliminates parasites such as *Leishmania, Toxoplasma*, and *Trypanosoma* (13, 23, 24). MIF can also be harmful to the host. That is, MIF production has been linked to pathology during malaria and *T. brucei* infection, by promoting inflammation-induced tissue damage (21, 25, 26). The role of host MIF during parasite infections has been well-reviewed elsewhere (23, 24, 27).

Counterintuitively, many pathogenic protozoans, including *Plasmodium*, *Entamoeba*, *Toxoplasma*, and *Leishmania*, produce their own MIF cytokine. These secreted parasite-produced MIF are structurally similar to human MIF, bind the MIF receptor (CD74), and stimulate immune cells and epithelial cells to cause the release of cytokines such as TNF-α, IL-8, and IL-12 (28–35). While it seems counterintuitive for protozoans to secrete a proinflammatory cytokine, it appears they have an important role in the parasite life cycle. Here, we focus on MIF produced by medically important protozoans, highlighting the recent contributions that have improved our understanding of the role of protozoan MIF in immune evasion, invasion, and pathogenesis (**Figure 1**).

IMMUNE EVASION

The host deploys a robust immune response to prevent parasite invasion, clear the infectious pathogen, and prevent re-infection. However, parasites have developed a remarkable number of mechanisms to evade these attacks (10). For example, *Leishmania* has developed ways to modify host cell signaling pathways, in order to survive and persist in host cells. *Leishmania* targets macrophages, which, interestingly, are the primary immune cells involved in the parasite's eradication (13). *Leishmania major* encodes two isoforms of MIF which facilitates its persistence in macrophages and contributes to its evasion from immune clearance. *L. major* MIF binds to CD74 on infected macrophages, activating the ERK1/2 pathway and preventing apoptosis of macrophages (35, 36). Infected macrophages then survive a sufficiently long enough time for the parasite to avoid excessive

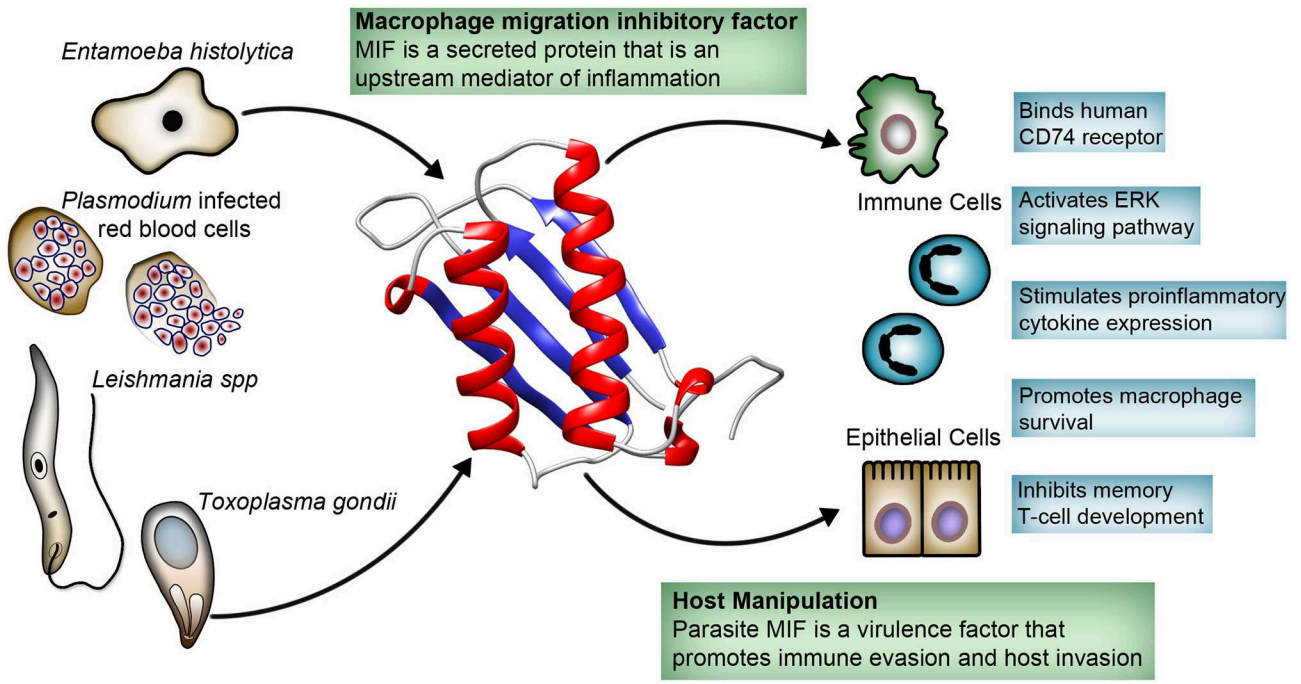

FIGURE 1 | Host-parasite interaction involving protozoa-produced macrophage migration inhibitory factor (MIF). Protozoa secrete MIF that is structurally similar to human MIF. Protozoa MIF binds directly to the human MIF receptor CD74, activating the ERK pathway with immunomodulatory effects on variety of immune and epithelial cells. Protozoa MIF immunomodulatory effects appear to play a role in parasite invasion and immune evasion, and has been linked to pathogenesis.

exposure to the immune system and complete its infectious life cycle.

The lack of protective immunity against re-infection is one of the biggest problems in controlling the transmission of protozoan infections. An adequate amount of protective memory T-cells are needed to fight off re-infection (37). Recent research in parasite MIF has provided a mechanism by which parasites evade the immune response by interfering with the development of immunological memory during infection, allowing them to re-infect their host (28, 29, 35). Using mouse models, researchers found that the proinflammatory effects of both *Plasmodium* and *Leishmania* MIF can manipulate T-cell differentiation. *Plasmodium* MIF enhances the production of IFN-γ and IL-12 which reduces the anti-*Plasmodium* blood-stage CD4 T-cell response. Mice infected with MIF-deficient *P. berghei* had reduced levels of these cytokines. This reduced inflammatory state correlated with improved survival of CD4 T helper cells. As a result, mice were able to develop effective T-cell memory when infected with MIF-deficient parasites which provided a protective response against a subsequent *P. berghei* infection. *Leishmania* MIF cause T-cells to develop into exhausted PD-1^+ short-lived effector cells with reduced IL-7R expression, which is needed to produce and maintain memory cells (28, 29, 35). These short-lived cells die during infection, and the long-lived memory T-cells required to prevent re-infection were not produced in adequate amounts (29). This MIF-induced lack of memory cells resulted in parasitic re-infection.

Recent clinical observations also support these findings. It was observed that in a cohort of children in an area endemic for amebiasis, those who lacked adequate amounts of antibodies against *E. histolytica* MIF were not protected from future infection (**Figure 2A**). The authors postulated that *E. histolytica* MIF might share similar properties to *Plasmodium* and *Leishmania* MIF. That is, *E. histolytica* MIF might also inhibit the development of sufficient amounts of memory cells. Thus, antibodies against *E. histolytica* MIF would block this effect resulting in adequate amounts of memory cells to protect against reinfection (32). Nevertheless, further studies are needed to confirm this theory. Also, the role of *Toxoplasma* MIF in immune evasion remains largely understudied.

INVASION

Host tissue invasion by extracellular or intracellular protozoan parasites play an important role in the pathogenesis of disease. The extent of tissue invasion by extracellular parasites correlates with the degree of disease severity (16). For example, the depth of tissue invasion is associated with worse outcomes in clinicopathological studies of patients with severe amebic colitis (38, 39). On the other hand, obligate intracellular parasites invade host cells to complete their life cycle (13, 40). Several parasite factors are known to contribute to the invasion process. Recent studies have implicated parasite MIF proteins in facilitating invasion and dissemination of several protozoan parasites.

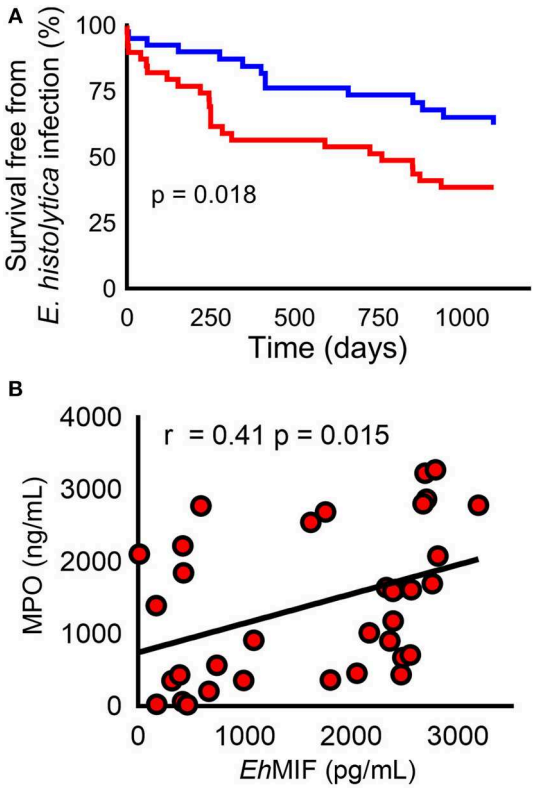

FIGURE 2 | *E. histolyica* MIF (*Eh*MIF) in human amebiasis. **(A)** Children in the top 50th percentile for anti- *Eh*MIF antibody (blue line) had a significantly higher probability of survival free of *E. histolytica* infection than children within lower 50th percentile (red line). **(B)** Significant positive correlation between fecal *Eh*MIF levels and the myeloperoxidase (MPO) marker of intestinal inflammation in persons with amebiasis ($n = 35$). Panels are reproduced from (17) with permission.

The extracellular matrix (ECM) is a network of proteins that provides tissue support and represents a major physical barrier to the parasite invasion. Matrix metalloproteinases (MMPs) are enzymes primarily responsible for ECM breakdown (41). Protozoa infections trigger an inflammatory response which leads to MMP overexpression, resulting in ECM breakdown. ECM degradation facilitates cell movement, and allows immune cell infiltration at the site of infection for host defense (42). Parasites have developed mechanisms to exploit the activities of MMPs to promote their invasion. For example, MMPs play a critical role in *E. histolytica* invasion. MMP expression is increased in human amebic colitis and inhibition of MMP prevented *E. histolytica* invasion in a human colonic explant model (32, 43, 44). Recently, a causal relationship between *E. histolytica*-produced MIF and gut inflammation was established using cellular and mouse models of amebic colitis. In the same study, researchers found that *E. histolytica* MIF-induced inflammation resulted in increased MMP production (32). Therefore, *E. histolytica* parasites appear to produce MIF as a virulence factor to exploit the inflammatory response to promote tissue invasion.

As mentioned above, neutrophils are the first immune cells to reach the site of *Leishmania* infection after a sand fly bite, and their uptake by neutrophils followed by macrophage engulfment contributes to leishmanial parasites infectivity and assist in life cycle progression (12–14). Neutrophils are short-lived phagocytes that act as a "Trojan horse" used by *Leishmania* parasites to obtain entry into macrophages thereby avoiding cell activation (15). Whether there are leishmanial factors that drive this neutrophil infiltration remains largely unanswered. While host MIF exhibits chemokine-like activities through interactions with the chemokine receptors CXCR2 and CXCR4 (45, 46), protozoan MIF-CXCR2 and CXCR4 interactions remain unclear.

Similar to *Leishmania*, *T. gondii* induces immune cell infiltration and not only evades their killing, but also hitches a ride in these cells to spread infection (47, 48). *T. gondii* must cross the intestinal barrier for it to advance from the gut to sites of secondary infection, and tachyzoites are often found in neutrophils in the gut lumen. *In vitro* studies have indicated that *T. gondii* MIF stimulates the production of the potent chemoattractant IL-8 from human cells. In an attempt to explain why this would benefit the parasite, it has been suggested that MIF-induced IL-8 production leads to neutrophil recruitment. Infected neutrophils, which are incapable of clearing the parasite, serve as motile reservoirs for *T. gondii* infection, facilitating the transepithelial migration of the parasite (30, 48, 49). While plausible, additional studies are warranted to validate the role of *Toxoplasma* MIF in invasion.

PATHOGENESIS

During protozoan infections, an unbalanced inflammatory reaction increases tissue destruction which leads to clinical disease. The inflammatory response is essential in that it provides protection against invading microbes. However, protozoan parasites have developed effective strategies to evade the immune response, avoid elimination, and persist in their host, which exacerbates the damage caused by the lingering inflammatory response to invading parasites (10). This is further compounded by the fact that these parasites secrete MIF cytokine that can directly drive inflammation.

Host cytokines released during *Plasmodium* infection contribute to severe malaria. For example, high TNF-α production is a strong predictor of severe malarial anemia and cerebral malaria in children (50, 51). *P. falciparum* MIF was shown to stimulate TNF-α secretion by immune cells *in-vitro*. Also, circulating serum *P. falciparum* MIF levels positively correlated with serum TNF-α levels in malaria patients, and higher *P. falciparum* MIF levels were observed in patients with severe malarial anemia and cerebral malaria (25, 28). These findings suggest that *P. falciparum* MIF is likely contributing to immunopathogenesis during malaria.

Neutrophil infiltration is a hallmark of amebic colitis. Neutrophils generate oxygen free radicals that are capable of killing the *E. histolyica* parasite. That said, *E. histolytica* has developed several strategies to counter and survive neutrophil killing (52–54). This results in an excessive and persistent

neutrophil response in the gut that has been shown to be associated with the most severe forms of human amebiasis, which also carry high fatality (6, 55–57). *E. histolyica* MIF plays an essential role in neutrophil infiltration during infection. *E. histolytica* MIF was shown to stimulate IL-8 and the murine IL-8 homolog KC (potent neutrophil chemoattractants), resulting in neutrophil infiltration and tissue destruction in cellular and mouse models. A recent human study found that gut *E. histolyica* MIF levels correlated with intestinal inflammation severity [**Figure 2B**; (32, 33)].

Macrophages also play a crucial role in protozoan MIF-induced immunopathology. *In vitro* studies show that *E. histolyica* MIF directly enhances TNF-α and IL-6 production from macrophages (31). Both cytokines cause collateral tissue injury in amebic colitis and liver abscess (10, 58). In a mouse model of *Leishmania* infection, *Leishmania* MIF upregulated inflammatory and innate immune signaling in infected macrophages, such as CXCL1, TLR2, and TNF-α, when compared to $MIF^{-/-}$ strains (35). Taken together, this pro-inflammatory phenotype, extending the survival of infected macrophages, and defective adaptive immune response supports the contribution of *Leishmania* MIF to the chronic destructive inflammatory state observed in leishmaniasis.

OTHER PROTOZOANS PRODUCING MIF

Other medically important protozoans include *Trichomonas, Giardia, Trypanosoma, Acanthamoeba*, and *Naegleria*. MIF orthologs have been discovered in *Trichomonas* and *Giardia*. The structure of *Giardia* MIF has been solved with a characterization similar to human MIF, but its role in infection is not well-understood (59). Surprisingly, MIF orthologs have not been characterized in *Trypanosoma*, *Acanthamoeba*, and *Naegleria*. However, incomplete genome assembly and annotation may limit *in-silico* analysis and explain why MIF has yet to be identified in these protozoans.

Inflammation is a critical component of tumor progression and many cancers, including prostate cancer, arise from sites of infection and chronic inflammation (60, 61). *Trichomonas vaginalis* is a sexually transmitted parasite that can colonize the prostate in men. *T. vaginalis* also secretes MIF which has pro-inflammatory properties. In addition to stimulating the production of IL-8 and IL-6 cytokines, *Trichomonas* MIF binds to the human CD74 MIF receptor triggering the activation of the pro-proliferative ERK and P13K/Akt pathways in prostate epithelial cells. *Trichomonas* MIF-driven inflammation and cell proliferation, was linked to the promotion and progression of prostate cancer (62).

CONCLUSION

Recent studies have made it increasingly clear that parasite-produced MIF is a virulence factor that play a significant role in host-parasite interactions and contributes to pathogenesis. Despite these advances, key questions remain unanswered. Such as, can we translate these findings to provide beneficial interventions to patients infected with these pathogens? Do we know enough to intervene in a meaningful way? Protozoan MIF (P-MIF) appears to be a logical candidate for further evaluation as an effective immunotherapeutic target given the accumulation of data showing that: (i) infected persons naturally make antibodies against P-MIF, (ii) anti-P-MIF do not cross-react with host MIF, and (iii) neutralizing antibodies inhibit P-MIF activity and therefore prevent re-infection and reduce immunopathology.

AUTHOR CONTRIBUTIONS

SG, NJ, LF, RN, and SM wrote different sections, edited, and reviewed the manuscript.

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Computational Network Analysis Identifies Evolutionarily Conserved miRNA Gene Interactions Potentially Regulating Immune Response in Bovine Trypanosomosis

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Bovine trypanosomosis is a devastating disease that causes huge economic loss to the global cattle industry on a yearly basis. Selection of accurate biomarkers are important in early disease diagnosis and treatment. Of late, micro-RNAs (miRNAs) are becoming the most useful biomarkers for both infectious and non-infectious diseases in humans, but this is not the case in animals. miRNAs are non-coding RNAs that regulate gene expression through binding to the 3′-, 5′-untranslated regions (UTR) or coding sequence (CDS) region of one or more target genes. The molecular identification of miRNAs that regulates the expression of immune genes responding to bovine trypanosomosis is poorly defined, as is the possibility that these miRNAs could serve as potential biomarkers for disease diagnosis and treatment currently unknown. To this end, we utilized *in silico* tools to elucidate conserved miRNAs regulating immune response genes during infection, in addition to cataloging significant genes. Based on the *p* value of 1.77E-32, we selected 25 significantly expressed immune genes. Using prediction analysis, we identified a total of 4,251 bovine miRNAs targeting these selected genes across the 3′UTR, 5′UTR and CDS regions. Thereafter, we identified candidate miRNAs based on the number of gene targets and their abundance at the three regions. In all, we found the top 13 miRNAs that are significantly conserved targeting 7 innate immune response genes, including bta-mir-2460, bta-mir-193a, bta-mir-2316, and bta-mir-2456. Our gene ontology analysis suggests that these miRNAs are involved in gene silencing, cellular protein modification process, RNA-induced silencing complex, regulation of humoral immune response mediated by circulating immunoglobulin and negative regulation of chronic inflammatory response, among others. In conclusion, this study identifies specific miRNAs that may be involved in the regulation of gene expression during bovine trypanosomosis. These miRNAs have the potential to be used as biomarkers in the animal and veterinary research community to facilitate the development of tools for early disease diagnosis/detection, drug targeting, and the rational design of drugs to facilitate disease treatment.

Keywords: cattle, miRNA-target, expression, trypanosomosis, evolution, immune response

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INTRODUCTION

Every year, the Cattle industry is faced with huge economic losses due to diseases such as trypanosomosis. Bovine trypanosomosis is endemic in sub-Saharan Africa and some parts of The Americas, causing losses of several billion dollars annually (Abdi et al., 2017; Odeniran and Ademola, 2018). The disease is associated with multifactorial complex phenotype, requiring an integrative biological approach to elucidate the molecular networks involved and to identify significant markers that may be useful for disease diagnosis and treatment (Noyes et al., 2011; Smetko et al., 2015; Kim et al., 2017). Recently, major research efforts are focused on utilizing microRNA (miRNA) as diagnostic biomarkers or therapeutic targets due to their involvement in various disease phenotypes and malignancies (Lawless et al., 2014; Scheel et al., 2017; Ammah et al., 2018; da Silveira et al., 2018). MicroRNAs are short, non-coding RNAs, which are capable of post-transcriptionally modifying gene expression, and form complexes with argonaute proteins that hybridize to the target mRNAs leading to the degradation of mRNA and negatively impact protein expression (Bushati and Cohen, 2007; Bartel, 2009; Meister, 2013; Huberdeau et al., 2017).

Post-transcriptional modification of gene expression is becoming a major focus in disease studies (Liu and Wang, 2019), with several reports showing miRNAs as important players in post-transcriptional regulation of both innate and adaptive immune gene expression (O'Connell et al., 2010; Sandhu et al., 2012; Kramer et al., 2015; Mehta and Baltimore, 2016; Pichulik et al., 2016; Kumar et al., 2018; Awuah et al., 2019). miRNAs have also been implicated in the regulation of immune functions in different cell types, for example neutrophil senescence and naïve mouse B cells have been reported to be regulated by repertoires of miRNAs (Ward et al., 2011; Bronevetsky et al., 2013; Aalaei-Andabili and Rezaei, 2016; Kangas et al., 2017). While there are numerous miRNAs abundantly expressed in bovine tissues and the entire genome, there are very few studies elucidating their regulatory role in bovine immunity (Coutinho et al., 2007; Jin et al., 2009; Xu et al., 2009; Hanif et al., 2018; Li et al., 2019). MicroRNAs therefore, will play important and unique roles during immune response, including modification of gene expression during bovine trypanosomosis. For instance, mir-2284 and mir-2285 families have been associated with TLR genes, thereby regulating non-specific immune responses through the production of pro-inflammatory cytokines, suggesting a role in bovine disease tolerance (Das et al., 2016). Similarly, mir-2404, mir-2285, and mir-6522 have been reported to regulate CD8 alpha chain and T-cell surface glycoprotein, thus playing significant role in development of T and B cell, proliferation, and inflammatory process (Mahjoub et al., 2015; Hanif et al., 2018). Studies have shown that miRNAs repress the target genes through the 3′ untranslated region (3′UTR) of mRNAs, therefore the miRNA binding site in this region seems to be well characterized (Buza et al., 2014; Liu and Wang, 2019). However, comparative genome analysis has shown that both coding domain sequence (CDS) and 3′UTR sites are under selective pressure while many miRNA binding sites are conserved in both 3′UTR and CDS (Hausser et al., 2013; Brummer and Hausser, 2014). In fact, many investigators are suggesting an alternative mode of gene regulation, whereby miRNAs anneal within the CDS, 5′- and/or 3′-UTR regions of their targets thereby regulating their translation.

Hence, there remains an important need to identify possible miRNA binding sites within the complete sequence of a gene (i.e., 5′-UTR, CDS and 3′-UTR), which can serve as potential biomarkers for disease diagnosis and treatment. To date, there has not been a published study identifying potential miRNAs as biomarkers in the entire sequence of immune genes responding during bovine trypanosomosis, demonstrating the uniqueness of this study. Notably, there are many miRNA databases and algorithms, which are available to investigate significant pathways and transcriptional mechanisms within various interaction and expression datasets (Xie et al., 2013; Li et al., 2014; Hanif et al., 2018). There are also proven *in silico* tools and algorithms available for miRNA target prediction and for dissecting miRNA function (Kogelman et al., 2014; Scheel et al., 2017; Hanif et al., 2018). A quantitative measurement of miRNA expression in conjunction with their target gene expression would be an important means to deconvolute diverse biogenesis pathways and immune system regulation during bovine trypanosomosis. To this end, we have employed a combination of computational tools to identify and elucidate conserved miRNAs, their target interaction(s), and regulatory function(s) during bovine trypanosomosis. This study, we believe, is a proof of concept with the potential to guide future studies aiming to identify attractive and putative miRNA targets for the rationale design and/or discovery of drugs to combat diseases, including potential biomarkers for disease diagnosis.

MATERIALS AND METHODS

Data Mining and Verification of Genes Associated With Bovine Trypanosomosis Pathway

In this study, we performed a data survey of genes that are reported to be significantly regulated during bovine trypanosomosis through publicly available databases. Briefly, we used LitInspector of Genomatix Literature Mining software (Version 3.10, Munich, Germany[1]) that uses proprietary literature data mining algorithms based on all available PubMed publications and their corresponding Medical Subject Headings (MeSH) (Berriz et al., 2003; Maier et al., 2005). This program gathers gene information and correlations with diseases and pathways from published literature. The gene sets are characterized based on annotation and literature including MeSH terms, gene ontology (GO) and pathways, as described (Scherf et al., 2005; Frisch et al., 2009). Out of the 3,738 disease terms found in the MeSH database, we selected only one

[1]https://www.genomatix.de/solutions/genomatix-software-suite.html

term relevant for our study, "trypanosomosis" (MeSH-Term ID: c03.752.300.900), using bovine as our reference species. The list of individual genes in association with bovine trypanosomosis as found in the scientific literature was filtered for significance to avoid random matches (Buza et al., 2014). Based on the parameters defined above, we selected a total of 25 genes that are significantly regulated and associated with our disease term based on the MeSH *p*-value (p = 1.77E-32). The 25 gene sets were then used as targets for further analysis (gene functions and references in **Supplementary Table S1**).

Prediction and Extraction of Disease Associated Bovine miRNA

We searched for the identified 25 genes from our disease pathway and their locations within the bovine genome UMD 3.1 using the Ensembl BLAST/BLAT Genomic Sequence tool[2]. Complete sequence of the 25 genes were retrieved with their accession number: CD86 (XM_005201387), FcγR3A (NM_001077402), CD1A (NM_001105456), IL-6 (NM_173923), IFN-G (NM_174086), CD4 (NM_001103225), CXCL-8 (NM_173923), ICAM-1 (NM_174348), CSF2 (NM_174028), CD14 (XM_005209429), TLR-4 (NM_174198), LBP (NM_001038674), IL-10 (NM_174088), TLR-2 (NM_174197), CCL2 (NM_174006), MYD88 (NM_001014382), TNF (NM_173966), CD83(NM_001046590), CD80 (NM_001206439), IL-4 (NM_173921), IL-18 (NM_174091), LY96 (NM_001046517), ITGAM (NM_001039957), IL-12A (NM_174355), and MAPKAPK3 (NM_001034779). Thereafter, each gene was used as target to individually search the *Bos taurus* genome for possible miRNAs within their complete sequences (i.e., 3′-UTR, 5′-UTR and CDS) using miRWalk[3]; an online program which allows search for interactions between complete gene sequences and miRNAs using the TarPmiR algorithm. To avoid false positive miRNAs in our study, three other miRNA-target prediction software (TargetScan[4], miRDB[5] and miRBase[6]) were used to predict the miRNA and targets interaction for each gene. Only miRNAs that were confirmed in the three databases were included for further analysis. All miRNAs that were found from each gene target were partitioned into three regions; the 5′-UTR, CDS and 3′-UTR in order to examine the variation in miRNA abundance at each region of the targets. In order to identify common miRNAs among the three regions of each gene sequence, we sorted the matches using a web- based Venn diagram application[7].

Network Analysis, Interaction of miRNA and Target Location of the Chromosome

Using the Ensembl BLAST/BLAT and the miRBase, we locate the position of each miRNA and their targets on the chromosomes within the bovine genome. All mature miRNAs sequences of our targets were retrieved; genes located on the same chromosome were pooled together for us to identify concomitant miRNAs co-regulating one or more targets with the aid of an online Venn diagram[8]. miRNA-target network was constructed with most significant genes in the disease pathway using miRNet; an online platform that integrate miRNAs, targets and their functions[9] (Fan and Xia, 2018).

Analysis of 7 Innate Immune Genes Associated With Bovine Trypanosomosis

We selected seven innate immune genes among the 25 that are responding in bovine trypanosomosis pathway for further analysis. Briefly, we used the accession numbers of TLR-4 (NM_174198), ITGAM (NM_001039957), ICAM-1 (NM_174348), CD14 (XM_005209429), TLR-2 (NM_174197), LBP (NM_001038674), and TNF-α (NM_173966) to query the databases of miRWalk[10] and miRBase[11] in order to retrieve the mature and precursor miRNA sequences within the targets for further analysis. We selected only common miRNAs from the two databases as candidates for further analysis. Based on the number of targets by a miRNA, we selected the top 10 miRNAs regulating four to five targets. It is assumed that a miRNA with multiple targets is probably a key miRNA in the innate immune system under study (Newman and Hammond, 2010; Wang, 2010). We also compared the miRNA abundance at 5′-UTR, CDS and 3′-UTR of the selected top 10. This is also necessary assuming the regions with the most miRNAs will be more regulated and have greater impact on disease outcome.

Evolutionary Trace and Phylogenetic Analysis of Conserved Bovine miRNA With Other Species

In order to determine the evolutionary trace and conservation of the identified bovine miRNA from the seven innate immune response genes, we selected the two miRNAs that showed the highest conservation from the 5′-UTR and 3′-UTR and 10 from the CDS being that it presented the most abundant miRNAs. Here, precursor miRNA sequences were retrieved from the miRBase database (see footnote 11); each bovine miRNA sequence was used for BLAST search with Ensembl BLAST/BLAT Genomic Sequence tool[12]. Homologous sequences from other species for each bovine miRNA were downloaded from the database for further analysis. Multiple sequence alignment (MAS) was performed using MEGA vs7 and Neighbor joining (NJ) phylogenetic tree was constructed with Cluster W to determine the nucleotide substitution and evolutionary divergence among the species. It is assumed that the most conserved miRNAs would be candidates for disease studies.

[2]http://www.ensembl.org/Tools/Blast/GenomicSeq

[3]http://mirwalk.umm.uni-heidelberg.de

[4]http://www.targetscan.org/vert_72/

[5]http://mirdb.org/

[6]http://www.mirbase.org/index.shtml

[7]http://bioinfogp.cnb.csic.es/tools/venny/

[8]http://bioinformatics.psb.ugent.be/software/details/Venn-Diagrams

[9]https://www.mirnet.ca/miRNet/faces/upload/GeneUploadView.xhtml

[10]http://mirwalk.umm.uni-heidelberg.de/search_genes/

[11]http://www.mirbase.org/index.shtml

[12]http://www.ensembl.org/Tools/Blast/GenomicSeq

Gene Ontology and Functional Analysis of the Most Conserved Bovine miRNAs in Innate Immune System

We performed functional enrichment analysis using gene ontology (GO) term[13] and DIANA tools[14] for the topmost conserved miRNAs at the 3′UTR, 5′UTR and CDS of the 7 innate immune response genes to gain understanding of the possible biological processes and physiological pathways regulated by these miRNAs according to Paraskevopoulou et al. (2013). We used 13 topmost miRNAs from the 7 innate immune genes and 6 others that are significantly conserved from CXCL-8, TLR-4, and MAPKAPK3 (**Supplementary Table S2**), giving a total of 19 miRNAs to search the gene ontology database to collect the relevant biological processes and the GO terms. A higher stringency was imposed by considering only the GO terms with $p < 0.001$ as significantly enriched. Where GO terms were not found for certain bovine miRNAs, closely related and annotated miRNAs in other species were used as functional homologs.

RESULTS

Identification of Candidate miRNAs and Gene Targets Associated With Bovine Trypanosomosis

Our data mining revealed a total of 25 genes which were significantly regulated during bovine trypanosomosis. From the prediction analysis, we identified 4,251 bovine miRNAs that target these 25 genes spanning the 3′UTR, 5′UTR and the CDS regions (**Table 1**). The number of miRNAs per gene ranges from 32 as seen in LY96 to 578 in MAPKAPK3 gene. As shown in **Figure 1** and **Table 1**, about 8 other genes showed a higher density with over 200 miRNAs. It is observed that ICAM-1 and ITGAM genes had similar miRNA number (220).

In order to understand the distribution of the miRNAs across the nucleotide sequence of each gene, we performed miRNA-target prediction for the 3′UTR, 5′UTR and the CDS regions using miRWalk. Generally, the CDS region presents the highest number of miRNAs for most genes followed by the 3′UTR, while the 5′UTR has the lowest (**Figure 2**). We observed that genes like LY96 and ITGAM have no miRNA at both 3′UTR and 5′UTR; CD80, IL-4 and IL-18 have no miRNA at the 3′UTR, with IL-12A, IFN-γ, IL-1- and MYD88 presenting no miRNA at the 5′UTR.

To examine the most conserved miRNAs across the 2 or 3 regions, we compared the overlapping miRNAs for each gene, with the assumption that miRNA present at two or three regions will be conserved with important biological inferences. Notably, there are more matches of miRNAs between the 3′UTR and CDS region with FcγR3A, CD14 and MAPKAPK3 genes presenting 18, 20 and 99 matches respectively while CD80, IL-4, IL-18, LY96, ITGAM and IL-12A had no match of miRNA at the 3 regions (**Table 2** and **Supplementary Table S2**). Overall, we observed that only CXCL-8, TLR-4, and MAPKAPK3 genes had conserved miRNAs present in their 3′UTR, 5′UTR and the CDS regions; bta-miR-2454-5p in CXCL-8, bta-miR-2328-3p in TLR-4, bta-miR-1777a, bta-miR-652 and bta-miR-7863 in MAPKAPK3 (**Supplementary Table S2**).

Conserved Bovine miRNAs With Different Chromosomes

We gathered miRNA from genes located on the same chromosome and we identified common miRNAs which may give biological relevance to our study. In all, we found a total of 15 out of 25 selected genes located on the same chromosome with chromosome 7 having 4 genes (CSF2, CD14, ICAM-1, and IL-4), followed by chromosome 1 possessing 3 genes (CD80, CD86, and IL-12A) while chromosomes 3, 5, 22 and 23 have 2 genes each in common (**Figures 3A–F**). MAPKAPK3 and MYD88 on chromosome 22 has the highest number of common miRNAs with a total of 130; while TNF-α and CD83 on chromosome 23 have 53 miRNAs in common. Thirty common miRNAs are presented between CD80, CD88, and IL-12A.

Network Analysis of Bovine miRNA-Target Interactome During Bovine Trypanosomosis

miRNA-target network was constructed in order to identify significant association and connections between miRNAs and different targets during bovine trypanosomosis. Out of the 25 selected genes and 4,251 miRNAs that were subjected to network analysis, only 13 genes and 54 miRNAs were significant connected in our network (**Figure 4**). Overall, based on the size of each node, CD14, MYD88, TNF-α, and IL-10 were significant nodes detected from the network analysis. Likewise, bta-mir-2888, bta-mir-2394, bta-mir-1284, and bta-mir-2467-p were major miRNA nodes connecting two or more genes in the network.

Prediction of Conserved miRNAs-Target Interaction in 7 Innate Immune Response Genes

We focused on identifying miRNAs that are concomitantly present in the 7 innate immune response genes among the list of 25 genes that are significantly responding to bovine trypanosomosis. Here, we postulate that bovine miRNAs targeting the greatest number of genes will be potential candidates as biomarkers for disease diagnosis or treatment. From **Tables 3–5**, we present the prediction analysis of miRNA-target interactions and their matured sequences. We showed the top 10 bovine miRNAs and their targets based on the 3′UTR, 5′UTR, and the CDS regions. Our selection is based on the miRNAs that target between 3-5 genes out of the selected 6 innate immune response genes. We observe that the CDS region has miRNAs that target the most genes; 5 out of the 7 genes at a time. For example, bta-miR-2349 targets CD14, ITGAM, TLR-2, TLR-4, and TNF-α while bta-miR-193a-5p targets ICAM-1, ITGAM, LBP, TLR-2, and TNF-α (**Table 3**). Following the CDS region is the 3′UTR, while the 5′UTR has the least number of

[13]http://geneontology.org/

[14]http://diana.imis.athena-innovation.gr/DianaTools/index.php

TABLE 1 | List of significantly regulated genes during bovine trypanosomiasis and the number of miRNA at the 3′UTR, 5′UTR and CDS regions.

S/No	miRNA Target	Accession #	Number of miRNA at 3′UTR	Number of miRNA at 5′UTR	Number of miRNA at CDS	Total miRNA	Chromosome number	Location on chromosome
1	CD86	XM_005201387	121	11	79	211	1	66542297–66612271
2	CD80	NM_001206439	0	3	108	111	1	64301045–64324140
3	IL-12A	NM_174355	1	0	265	266	1	107432816–107440698
4	FcγR3A	NM_001077402	92	4	116	212	3	8000142–8008338
5	CD1A	NM_001105456	28	5	100	133	3	11858509–11863336
6	IL-6	NM_173923	13	13	72	98	4	31454662–31459218
7	IFN-γ	NM_174086	32	0	57	89	5	45624365–45629433
8	CD4	NM_001103225	129	4	103	236	5	103630890–103655348
9	CXCL8	NM_173923	82	8	37	127	6	88810335–88814655
10	ICAM-1	NM_174348	81	39	100	220	7	14813516–14824624
11	CSF-2	NM_174028	23	2	60	85	7	22398891–22401350
12	CD14	XM_005209429	108	13	95	216	7	51762838–51765825
13	IL-4	NM_173921	0	4	65	69	7	21696091–21704293
14	TLR-4	NM_174198	40	43	154	237	8	107057606–107069056
15	LBP	NM_001038674	27	3	129	159	13	67214225–67247921
16	LY96	NM_001046517	0	0	32	32	14	37240179–37274547
17	IL-18	NM_174091	0	32	36	68	15	22475462–22502857
18	IL-10	NM_174088	25	0	70	95	16	4550747–4555407
19	TLR-2	NM_174197	50	12	135	197	17	3953755–3967506
20	CCL-2	NM_174006	31	10	51	92	19	15902726–15905419
21	MYD88	NM_001014382	126	0	48	174	22	11609261–11613877
22	MAPKAPK3	NM_001034799	284	42	252	578	22	49711038–49740804
23	TNF-α	NM_173966	68	22	96	186	23	27716111–27719104
24	CD83	NM_001046590	82	7	51	140	23	42654473–42676909
25	ITGAM	NM_001039957	0	0	220	220	25	27343250–27382467

Significantly regulated genes were arranged in order of chromosome number (lowest to the highest).

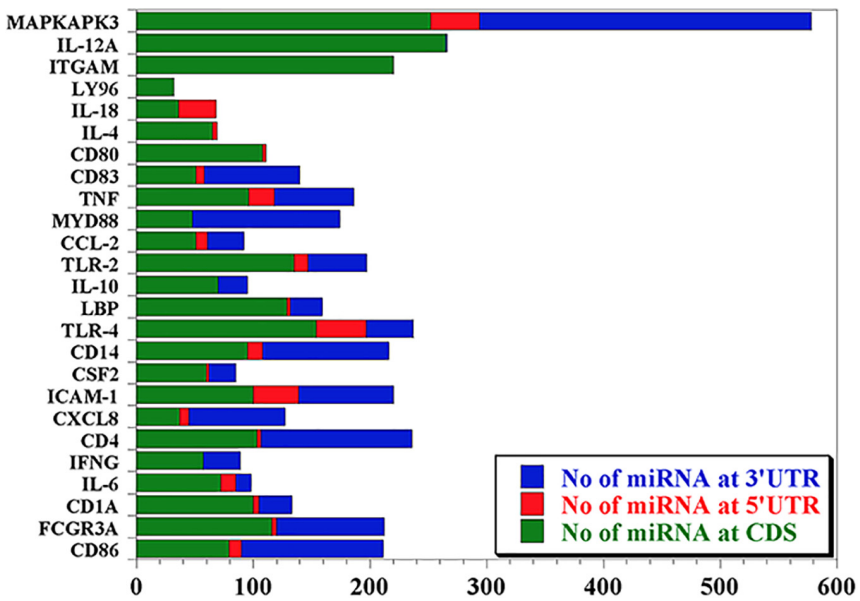

FIGURE 1 | Predicted miRNA abundance targeting 25 genes significantly regulated during bovine trypanosomosis.

miRNAs, targeting less than 4 genes (**Tables 4**, **5**). bta-miR-2460 from the 3′UTR is demonstrated to target CD14, ICAM-1, TLR-4, and TNF-α genes, while bta-miR-2374 are targeting CD14, ICAM-1, LBP, and TLR-4 gene sets (**Table 4**), indicating they are the topmost in this region. Only bta-miR-2392 is targeting ICAM-1, TLR-2, TLR-4 and TNF-α from the 5′UTR (**Table 5**).

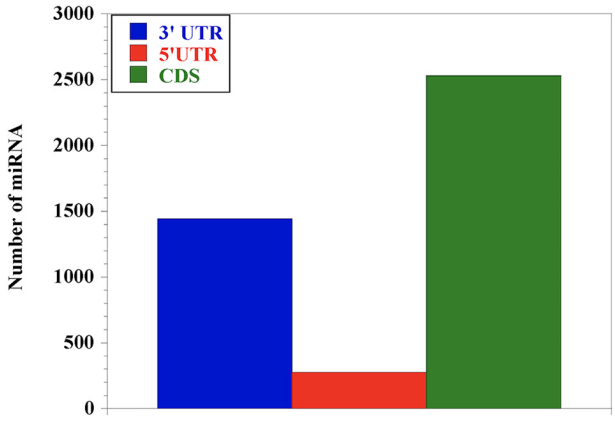

FIGURE 2 | Distribution of miRNA abundance at the 3′UTR, 5′UTR and CDS of the 25 genes responding during bovine trypanosomosis. Our results show that miRNAs are relatively abundant in the coding sequence (CDS) region, indicating the importance of this region in gene regulation, followed by the 3′UTR. The 5′UTR had the least number of miRNAs.

Table 6 shows the number of miRNAs common to a gene pair from each region. Our analyses reveal that the highest number of bovine miRNAs common between two genes are located at the CDS region. There are 55 miRNAs shared between TLR-4 and ITGAM, 54 between LBP and ITGAM, 52 between TLR-2 and ITGAM, 44 between ITGAM and ICAM-1, and 40 between TNF-α and ITGAM. Notably, CD14 and TNF-α have the highest common miRNA (15) at the 3′UTR followed by 10 between CD14 and TLR-4 (**Table 6**).

Study of Evolutionarily Conserved Bovine miRNA Among the 7 Innate Immune Response Genes and Other Species

Phylogenetic analyses were performed to examine the evolutionarily conserved bovine miRNAs with other species. To be more stringent, only miRNAs that target 4 out of 7 innate immune response genes were included in the analysis (one from the 5′UTR, 2 from 3′UTR and 10 from the CDS). Analysis of all the 10 miRNAs at the CDS with their homologous sequences from other species identified bta-mir-193a as highly conserved with human, monkey, dog, pig, chicken, gorilla, mouse, rat, rabbit, and alligator (**Figure 5**). In the same manner, bovine bta-mir-4657 clusters with a human homolog hsa-mir-4657, bta-mir-2888-2 with hsa-mir-6803 and bta-mir-2349 with hsa-mir-4769 (**Figure 5**). Furthermore, we individually searched for the conserved homologs of the 13 miRNAs selected for stringency (one from the 5′UTR, 2 from 3′UTR, and 10 from the CDS) in order to determine their evolutionary trace with other species. **Figures 6A–M** show different clusters of each miRNA with their homologs from other species. Based on the number of homologs found with other species, bta-mir-2888-1 (**Figure 6J**) with 14 homologs has the highest followed by bta-mir-193a with 13 (**Figure 6A**), bta-mir-2888-2, bta-mir-2392, and bta-mir-2349 have 11, 9 and 8 homologs respectively (**Figures 6E,G,K**). bta-mir-2422 presented the least homolog with rat (rno-mir-327) and mouse (mmu-mir-327) (**Figure 6H**).

TABLE 2 | Overlapping bovine miRNAs at the 3′UTR, 5′UTR and CDS.

S/No	Gene	3′UTR 5′UTR match	3′UTR CDS match	5′UTR CDS match	3′UTR 5′UTR CDS match
1	CD86	3	13	1	0
2	FcγR3A	1	18	0	0
3	CD1A	2	6	0	0
4	IL-6	0	2	1	0
5	IFN-γ	0	7	0	0
6	CD4	0	13	1	0
7	CXCL8	1	10	0	1
8	ICAM-1	2	12	6	0
9	CSF-2	0	2	0	0
10	CD14	6	20	0	0
11	TLR-4	4	7	13	1
12	LBP	0	5	1	0
13	IL-10	0	4	0	0
14	TLR-2	0	7	4	0
15	CCL-2	0	2	2	0
16	MYD88	0	7	0	0
17	TNF	2	11	3	0
18	CD83	0	5	1	0
19	CD80	0	0	0	0
20	IL-4	0	0	0	0
21	IL-18	0	0	5	0
22	LY96	0	0	0	0
23	ITGAM	0	0	0	0
24	IL-12A	0	0	0	0
25	MAPKAPK3	18	99	10	3

UTR, untranslated region; CDS, coding sequence; red, indicate genes with matches and the numbers at the 3′UTR, 5′UTR and CDS regions.

Enrichment Analysis and GO Terms of the Conserved miRNAs

In order to better elucidate the possible biological and physiological processes regulated by the identified bovine miRNAs, 19 predicted conserved miRNAs were subjected to GO term analysis using the sub-term biological process as the focus. Topmost GO terms were selected for each tested miRNA as significantly enriched based on the *p*-value of less than 0.001. From the analysis, we found only 10 out of the 19 bovine miRNAs being functionally annotated with GO terms (**Table 7**). We infer the functional annotation of the remaining 9 bovine miRNAs from closely related species (in parentheses) based on the homology between 95-100%. We found that bta-mir-2460, bta-mir-193a, bta-mir-2328-3p, bta-mir-652, bta-mir-2392, and bta-mir-2454 are significantly involved with gene silencing (GO:0035195) and RNA-induced silencing complex (GO:0016442); bta-mir-2295 is involved with signal transduction in response to DNA damage (GO:0042770); bta-mir-2422 is involved with regulation of humoral immune response mediated by circulating immunoglobulin (GO:0002923), and bta-mir-2888-2 is involved with 2-aminobenzenesulfonate metabolic process (GO:0018868), among others (**Table 7**).

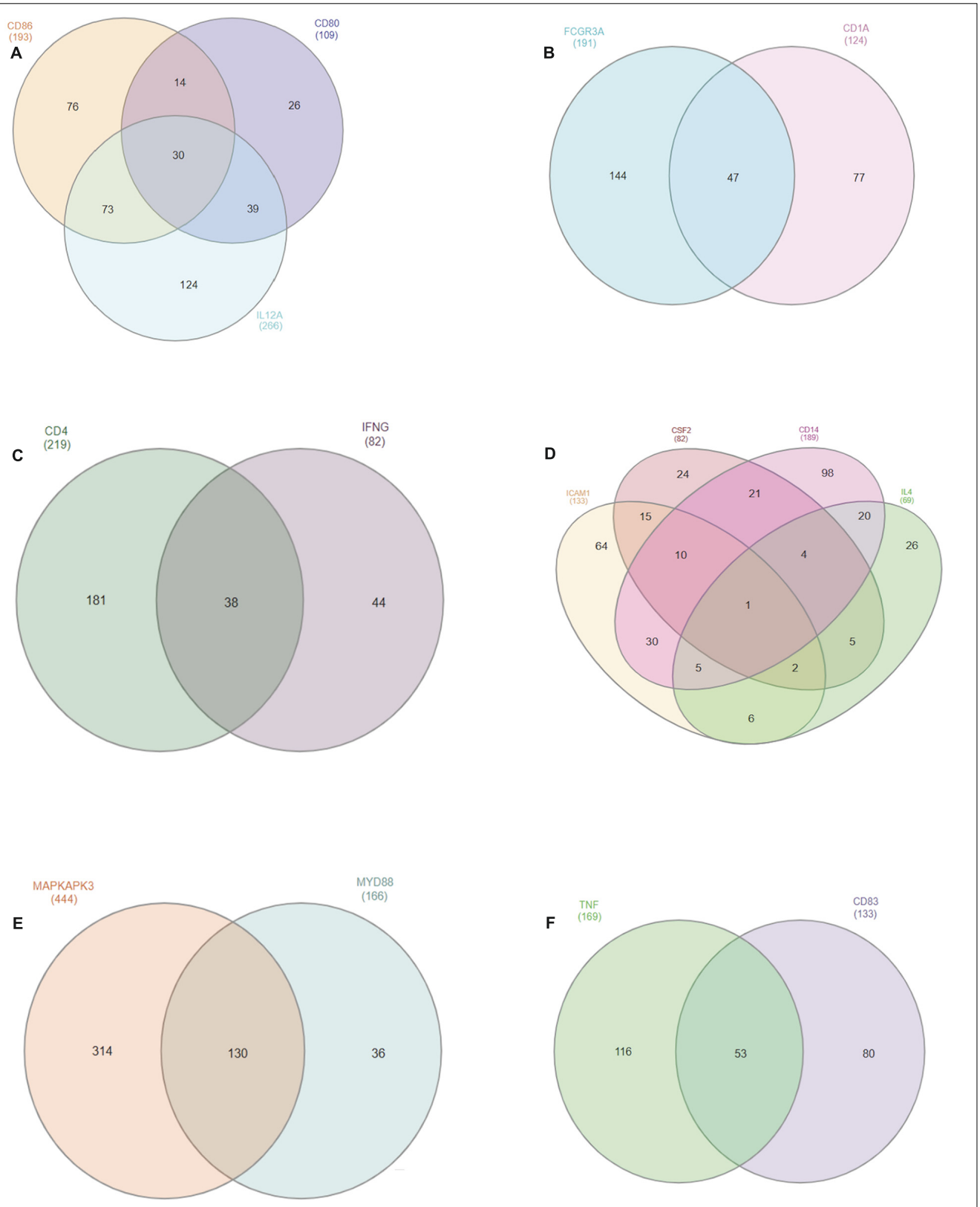

FIGURE 3 | Venn diagram of conserved bovine miRNAs within different chromosomes (**A:** chromosome 1; **B:** chromosome 3; **C:** chromosome 5; **D:** chromosome 7; **E:** chromosome 22; **F:** chromosome 23). Overlapping regions in the Venn diagram represent the number of conserved miRNAs between one or two genes in the same chromosome.

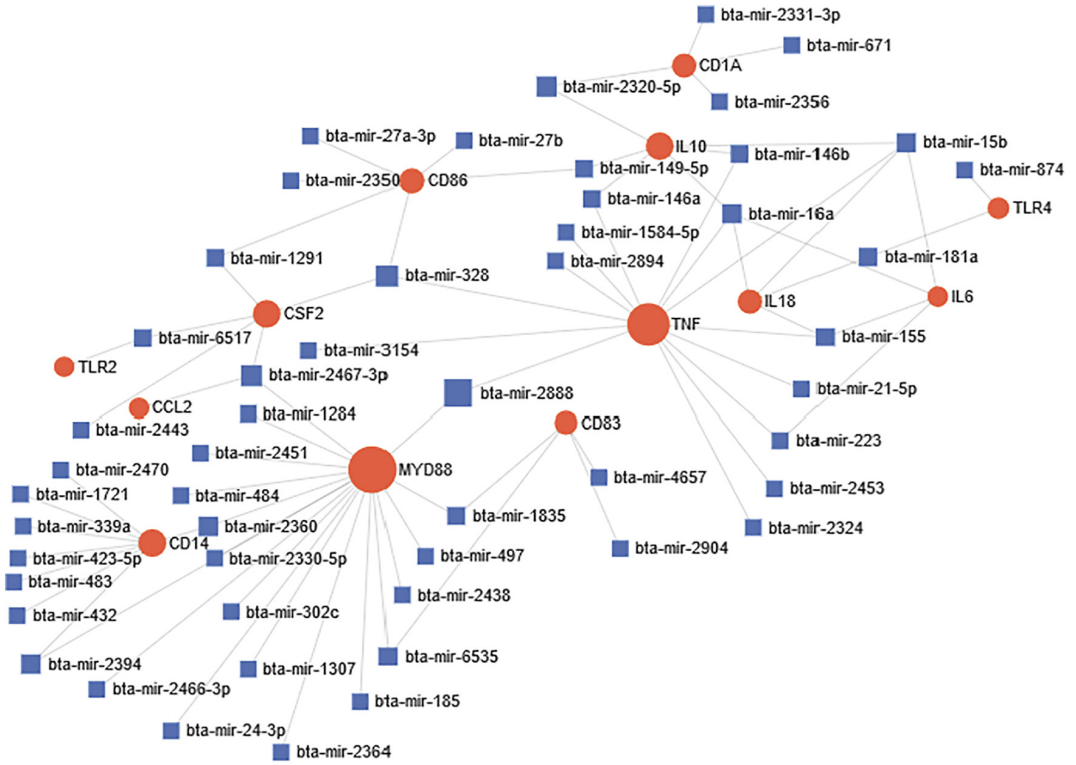

FIGURE 4 | Predicted miRNA-target interactome network during bovine trypanosomosis. Circular node shape with red color represents all significantly connected genes within the network during the disease condition. Node size simulates the number of interactions, which is directly proportional. Each miRNA in the network is represented by square shape and color blue. Most important miRNA nodes joined one or two genes together thereby affecting their expression simultaneously.

TABLE 3 | Top 10 MicroRNA at the CDS region and their target genes.

S/No	MicroRNA	Mature accession	Mature sequence	Size	Combined target genes
1	bta-miR-2349	MIMAT0011884	UGGCACUUCUGGUCUCAGACUCA	38–60	CD14, ITGAM, TLR-2, TLR-4, TNF
2	bta-miR-3602	MIMAT0016937	GUGUUGGGAUCACCGCGGUAA	18–38	CD14, ITGAM, LBP, TLR-2, TLR-4
3	bta-miR-449d	MIMAT0011962	GAAGGCUGUGUGCUGUGGAG	16–35	CD14, ITGAM, LBP, TLR-2, TLR-4
4	bta-miR-2316	MIMAT0011837	ACUCCGGCCUGGACUGCGGCGGG	10–32	CD14, ICAM-1, TLR-2, TLR-4, TNF
5	bta-miR-4657	MIMAT0036970	AAUGUGGAAGUGGUCUGAGGCAU	1–23	CD14, ICAM-1, LBP, TLR-2, TNF
6	bta-miR-2888	MIMAT0013846	GGUGGGGUGGGGGGGUUGG	47–65	ITGAM, LBP, TLR-2, TLR-4, TNF
7	bta-miR-2295	MIMAT0011803	UCGGGGUGGGAGGAAGGUUCU	43–63	ITGAM, LBP, TLR-2, TLR-4, TNF
8	bta-miR-193a-5p	MIMAT0003795	AACUGGCCUACAAAGUCCCAGU	49–70	ICAM-1, ITGAM, LBP, TLR-2, TNF
9	bta-miR-2422	MIMAT0011989	UUGAGGGGACUGAGGUGCGGAG	4–25	ICAM-1, ITGAM, LBP, TLR-2, TNF
10	bta-miR-2456	MIMAT0012041	ACGCACUGUCCUGGGAAGUGG	45–65	ICAM-1, ITGAM, LBP, TLR-4, TNF

DISCUSSION

Identification of molecular biomarkers associated with diseases is important in early disease diagnosis, drug target and treatment. miRNAs are increasingly emerging as molecular biomarkers because of their essential role in gene regulation both in animals and plants during disease condition (Buza et al., 2014; Agarwal et al., 2015; Scheel et al., 2017). In this study, we identified and elucidated conserved miRNAs from a list of gene targets important in innate immune response during bovine trypanosomosis through computational prediction analysis. Previous studies have focused on the 3′UTR region to study the miRNA-target interactions while few others suggested that miRNAs can anneal within the 3′UTR, 5′UTR or CDS regions of their target to regulate gene expression (Erhard et al., 2014; Yang et al., 2017; da Silveira et al., 2018). This study however brings a unique perspective through identification of conserved miRNAs targeting immune genes responding during bovine trypanosomosis by analyzing the complete sequence of the gene which include the 3′-, 5′ UTRs and the CDS. Our study showed a significantly higher density of miRNA binding sites at the CDS region for all genes, identifying it as an important region which may play a significant regulatory role in expression during the disease condition. Studies have shown

TABLE 4 | Top 10 MicroRNA at the 3′ untranslated region and their target genes.

S/No	MicroRNA	Mature accession	Mature sequence	Size	Combined target genes
1	bta-miR-2460	MIMAT0012045	UGGAGCUCUUGAGGCCUGGCAU	6–27	CD14, ICAM-1, TLR-4, TNF
2	bta-miR-2374	MIMAT0011920	UUGGGGCUGGGGAGAGGCGGG	45–65	CD14, ICAM-1, LBP, TLR-4
3	bta-miR-3431	MIMAT0017394	CCUCAGUCAGCCUUGUGGAUGU	21–42	CD14, ICAM-1, TLR-2
4	bta-miR-2343	MIMAT0011878	AAGGGGAGACGGUGGAACUUAU	11–32	CD14, ICAM-1, TLR-4
5	bta-miR-2308	MIMAT0011820	UUGGGCUUGCAGCAGAGAGUAA	44–65	CD14, ICAM-1, TLR-4
6	bta-miR-2328-3p	MIMAT0011857	GCCCCCUCCCUUGGUCGCCGG	10–30	CD14, TLR-4, TNF
7	bta-miR-328	MIMAT0009287	CUGGCCCUCUCUGCCCUUCCGU	61–82	CD14, TLR-4, TNF
8	bta-miR-2888	MIMAT0013846	GGUGGGGUGGGGGGGUUGG	47–65	CD14, LBP, TLR-4
9	bta-miR-3141	MIMAT0024573	GAGGGCGGGUGGAGGAGG	77–94	CD14, ICAM-1, LBP
10	bta-miR-320b	MIMAT0011991	AGCUGGGUUGAGAGGGUGGU	43–62	CD14, LBP, TNF

TABLE 5 | Top 10 MicroRNA at the 5′ untranslated region and their target genes.

S/No	MicroRNA	Mature accession	Mature sequence	Size	Combined target genes
1	bta-miR-2392	MIMAT0011945	AUGGAUGGGGGUGAGGGGUGCA	45–66	ICAM-1, TLR-2, TLR-4, TNF
2	bta-miR-181b	MIMAT0003793	AACAUUCAUUGCUGUCGGUGGGUU	36–59	CD14, TLR-2
3	bta-miR-1249	MIMAT0009976	ACGCCCUUCCCCCCCCUUCUUCA	41–62	CD14, ICAM-1
4	bta-miR-6120-5p	MIMAT0024590	CUGUUCCCGUUUUUCACAUGUG	3–24	CD14, TNF
5	bta-miR-329b	MIMAT0009289	AGAGGUUUUCUGGGUUUCUGUUU	13–35	CD14, TNF
6	bta-miR-27a-5p	MIMAT0012532	AGGGCUUAGCUGCUUGUGAGCA	14–35	TLR-2, TNF
7	bta-miR-345-3p	MIMAT0012535	CCUGAACUAGGGGUCUGGAG	55–74	ICAM-1, TLR-4
8	bta-miR-7865	MIMAT0030450	CAGGGAGGGCAGGGGAGGG	1–19	TLR-4, TNF
9	bta-miR-6526	MIMAT0025556	UCCUGUGCCUCGAAUGGGUAUG	48–69	TLR-4, TNF
10	bta-miR-2443	MIMAT0012018	UGAGGGCAGGACCGUAUGAGGUGU	9–32	LBP, TLR-4

TABLE 6 | Number of concomitant miRNAs at the 3′UTR, 5′UTR and CDS regions of seven innate immune response genes.

Gene target		CD14	TLR-2	TLR-4	ITGAM	ICAM-1	TNF-α	LBP
CD14	3′ UTR	71	6	10	0	12	15	5
	5′ UTR	9	1	0	0	1	2	0
	CDS	27	23	26	34	17	23	17
TLR-2	3′ UTR		27	1	0	9	8	1
	5′ UTR		9	1	0	1	2	0
	CDS		31	33	52	26	29	36
TLR-4	3′ UTR			20	0	7	6	6
	5′ UTR			37	0	2	3	2
	CDS			42	55	24	35	33
ITGAM	3′ UTR				0	0	0	0
	5′ UTR				0	0	0	0
	CDS				57	44	40	54
ICAM-1	3′ UTR					48	7	7
	5′ UTR					34	3	0
	CDS					15	22	27
TNF-α	3′ UTR						37	4
	5′ UTR						14	0
	CDS						13	32
LBP	3′ UTR							10
	5′ UTR							1
	CDS							28

that target genes having many miRNA binding sites at the CDS region can be easily degraded (Schnall-Levin et al., 2011; Hausser and Zavolan, 2014). Likewise, Ott et al. (2011) and Chi et al. (2009) discovered that there are increasing miRNA target sites at the CDS region of mammalian transcripts during immunoprecipitation studies. This observation is in line with our results, suggesting that CDS sites may have specific function in regulating gene expression rather than the previous focus on the 3′UTR (Hausser et al., 2013). Brummer and Hausser (2014) stated that the CDS sites may enhance the repertoire of miRNA regulation than the 3′UTR sites because of their interaction with alternative polyadenylation, alternative splicing and the general gene architecture.

In addition, our study identified significant overlaps of miRNA binding sites between the 3′UTR and CDS regions, indicating possible evolutionary conservation of these regulatory elements between the two regions (Hurst, 2006; Gaidatzis et al., 2007; Forman et al., 2008). Published reports have shown that miRNAs simultaneously targeting both 3′UTR and CDS regions of mRNAs are more regulated than those targeted by 3′UTR only (Fang and Rajewsky, 2011; Bazzini et al., 2012). Therefore, we adjudge that conserved miRNAs found targeting both regions have undergone selective pressure, and are therefore evolutionarily conserved, which may induce mRNA translational inhibition and degradation of immune gene response during bovine trypanosomosis.

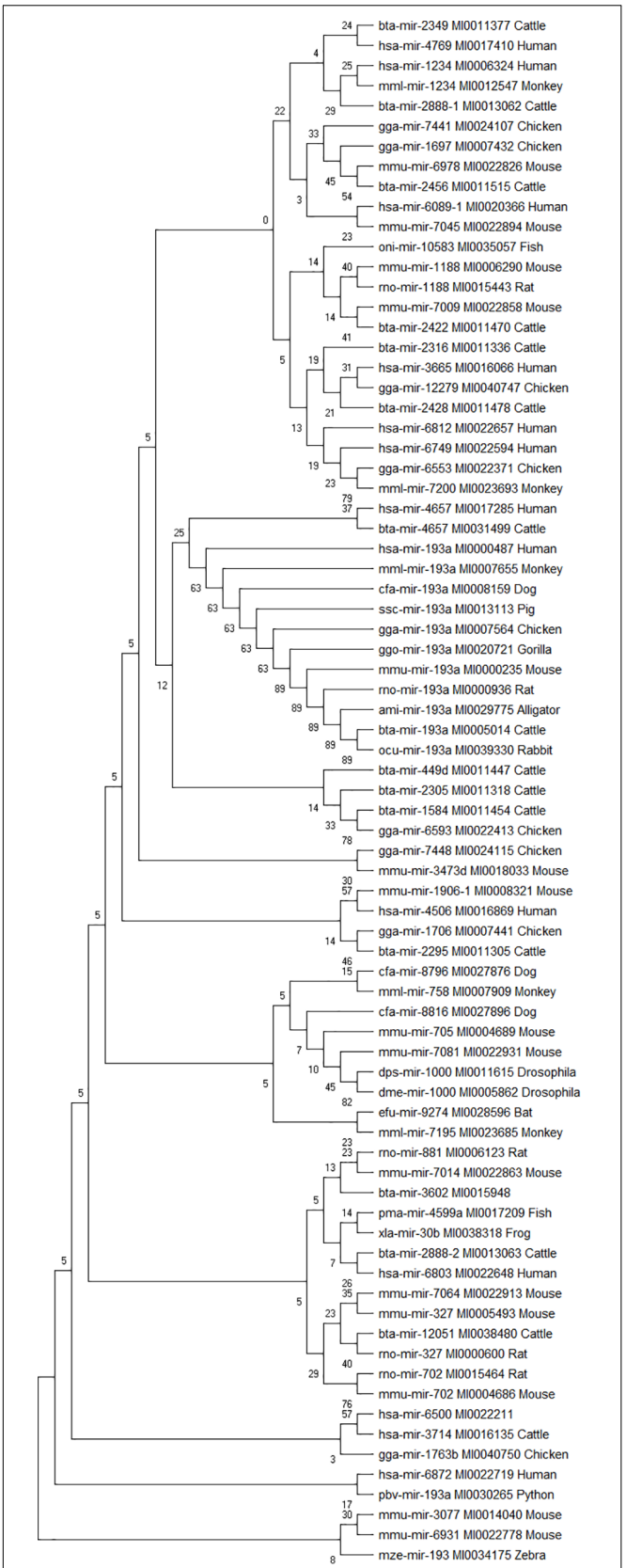

FIGURE 5 | Continued

FIGURE 5 | Phylogenetic tree of 13 bovine miRNA and their homologs in other species. The evolutionary trace was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of nucleotide sequence differences per site. The analysis involved 77 nucleotide sequences.

Furthermore, we found significant miRNAs cluster for genes located on the same chromosome, the most indication suggesting evolutionary conservation and functional roles on their targets. Thus, there is the possibility that expression of two or more genes on the same chromosome can be co-regulated by a single miRNA, and this observation might be important for quantifying gene regulation during disease condition or as targets for treatment. Pande et al. (2018) and Hanif et al. (2018) support the finding that miRNAs clusters across cattle genome have variable functions through large evolutionary distance.

Additionally, our study showed networks between miRNAs and target genes, which are significantly connected during bovine trypanosomosis. From our network interactome, CD14, TLR-2, TLR-4, IL10 MYD88, TNF-α, bta-mir-2360, bta-mir-1835, bta-mir-2888, bta-mir-146b and bta-mir-2320-5p amongst others are important drivers and connectors within the network, indicating they are major players during disease condition. Studies have shown that molecular interactions between genes, proteins, RNA and other biological molecules contribute to cellular performance and homeostasis (Govindaraju et al., 2012; Ji et al., 2018; Wang et al., 2018). The co-expression of these miRNAs within the network may regulate the interaction of certain gene sets such as MYD88 and TNF-α, CD14 and TLR-2, IL-18, and TLR-4, among others. Studies to date have demonstrated the importance of CD14 and TLR gene family in facilitating pattern recognition of pathogen-associated molecules and to elicit innate immune response against viruses, bacteria, fungi, and protozoa (Seabury et al., 2010; Ojurongbe et al., 2019; Morenikeji and Thomas, 2019b). Likewise, MYD88 is a signal transduction adaptor, transferring signals from interleukin-1 (IL-1) receptors and Toll-like receptors, and driving macrophage release of cytokines, imperative in innate immune response to exogenous pathogenic stimuli. Therefore, a significant overlap of miRNAs targeting these genes altogether could work synergistically as putative markers for disease discovery, characterization and therapeutic manipulations for bovine trypanosomiasis treatment.

Moreover, among the 7 innate immune response genes considered in this study, our functional analysis show that bta-mir-2460, bta-mir-193a, bta-mir-2349, bta-mir-3602, bta-mir-2454, and bta-mir-652 are significantly enriched within biological processes such as gene silencing, RNA-induced silencing complex, and cellular protein modification process. These miRNAs can potentially regulate CD14, ITGAM, LBP, TLR-2, TLR-4, and TNF-α genes and their expression might be key regulatory factors that determine disease susceptibility, tolerance or resistance in cattle. We have shown that CD14 is particularly implicated in disease tolerance among cattle with bovine trypanosomosis, with significant expression

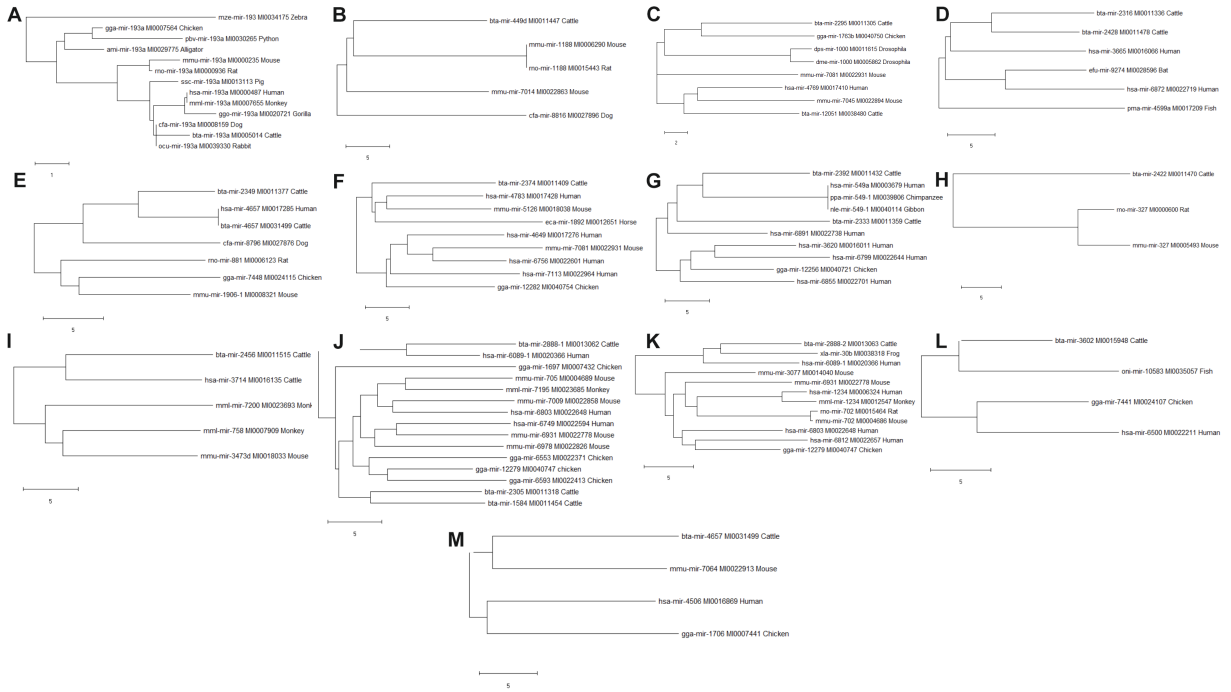

FIGURE 6 | Evolutionarily trace of conserved bovine miRNA among the 7 innate immune genes and other species (**A:** bta-mir-193a; **B:** bta-mir-449d; **C:** bta-mir-2295; **D:** bta-mir-2316; **E:** bta-mir-2349; **F:** bta-mir-2374; **G:** bta-mir-2392; **H:** bta-mir-2422; **I:** bta-mir-2456; **J:** bta-mir-2888-1; **K:** bta-mir-2888-2; **L:** bta-mir-3602; **M:** bta-mir-4657).

TABLE 7 | Gene Ontology of the top conserved predicted miRNA-target interaction.

miRNA	Regional location	GO term (biological process)	GO ID	*P*-value	Target genes
bta-mir-2374	3′UTR (human)	Cellular nitrogen compound metabolic process	GO:0034641	5.817e-07	CD14, ICAM-1, LBP, TLR-4
bta-mir-2460	3′UTR	Gene silencing by miRNA	GO:0035195	2.638e-03	CD14, ICAM-1, TLR-4, TNF
bta-mir-2392	5′UTR	RNA-induced silencing complex	GO:0035068	8.283e-05	ICAM-1, TLR-2, TLR-4, TNF
bta-mir-193a	CDS	Gene silencing by miRNA	GO:0035195	0.0076	CD14, ITGAM, TLR-2, TLR-4, TNF
bta-mir-449d	CDS (mouse)	Cellular differentiation	GO:0030154	0.0014	CD14, ITGAM, LBP, TLR-2, TLR-4
bta-mir-2295	CDS (chicken)	Signal transduction in response to DNA damage	GO:0042770	0.0018	CD14, ITGAM, LBP, TLR-2, TLR-4
bta-mir-2316	CDS (human)	Regulation of translation in response to stress	GO:0043555	0.0168	CD14, ICAM-1, TLR-2, TLR-4, TNF
bta-mir-2349	CDS (human)	Cellular protein modification process	GO:0006464	0.0373	CD14, ICAM-1, LBP, TLR-2, TNF
bta-mir-2422	CDS (human)	Regulation of humoral immune response mediated by circulating immunoglobulin	GO:0002923	0.0479	ITGAM, LBP, TLR-2, TLR-4, TNF
bta-mir-2456	CDS (mouse)	Cellular protein modification process	GO:0006464	7.549e-07	ITGAM, LBP, TLR-2, TLR-4, TNF
bta-mir-2888-1	CDS	Dihydrosphingosine-1-P pathway	GO:0006648	0.0069	ICAM-1, ITGAM, LBP, TLR-2, TNF
bta-mir-2888-2	CDS	2-aminobenzenesulfonate metabolic process	GO:0018868	2.720e-03	ICAM-1, ITGAM, LBP, TLR-2, TNF
bta-mir-3602	CDS (human)	Transcription regulator activity	GO:0010467	3.066e-05	ICAM-1, ITGAM, LBP, TLR-4, TNF
bta-mir-4657	CDS (human)	Cellular protein modification process	GO:0006464	0.0028	CD14, ITGAM, TLR-2, TLR-4, TNF
bta-mir-2454	3′UTR 5′UTR CDS	RNA-induced silencing complex	GO:0016442	7.857e-05	CXCL-8
bta-mir-2328-3p	3′UTR 5′UTR CDS	Gene silencing by miRNA	GO:0000115	0.0062	TLR-4
bta-mir-1777a	3′UTR 5′UTR CDS	Micro-ribonucleoprotein complex	GO:0016442	0.0012	MAPKAPK3
bta-mir-652	3′UTR 5′UTR CDS	Gene silencing by miRNA	GO:0035195	0.0016	MAPKAPK3
bta-mir-7863	3′UTR 5′UTR CDS	Negative regulation of chronic inflammatory response	GO:0008283	0.0887	MAPKAPK3

among trypanotolerant animals and the reverse among trypanosusceptible ones (Morenikeji and Thomas, 2019a). Notably, some studies have also shown that bta-mir-2460, bta-mir-193a, and bta-mir-652 are significantly involved in biological regulation, cellular and metabolic process, cell death, establishment of localization, and growth, thereby supporting their significant role in disease development (Jin et al., 2013; Schanzenbach et al., 2017; Li et al., 2018).

The significant number of targets by each miRNA depicts evidence of their evolutionary conservation with the implication that they play an essential role in gene regulation during bovine trypanosomosis. Yang et al. (2012) and Zheng et al. (2014) have reported the significant role of miRNAs in fine-tuning innate immune gene expression in Holstein cows during heat stress. Remarkably, bta-mir-2888-1 and bta-mir-2888-2 together targets ICAM-1, ITGAM, LBP, TLR-2, and TNF-α to respond to dihydrosphingosine-1-P pathway and 2-aminobenzenesulfonate metabolic process, which are important regulators of physiological biosynthesis, cell survival, proliferation, and cell-cell interactions (Bu et al., 2008). Previous studies have shown that ICAM-1, TLR-4 and ITGAM were implicated in early inflammation and signaling in critical pathways regulating bovine innate immunity (Boulougouris et al., 2019). As seen from our study therefore, bta-mir-2888-1 and bta-mir-2888 would be significant biomarkers that regulate the expression of these genes during disease condition and could serve as potential therapeutic targets for bovine trypanosomosis.

Our phylogenetic analysis revealed bta-mir-193a to be highly conserved across different species ranging from human to gorilla, monkey, mouse, rat, rabbit, chicken, pig, dog, python, and zebra. This shows that bta-mir-193a has a long history of evolution among others and would be a molecular marker of choice in bovine disease studies, and as such require additional *in vitro* validation studies. Reports from other studies have shown that bta-miR-193a regulates the expression of many proinflammatory cytokines, promote apoptosis and inhibition of bacteria in mice, goat and cattle (Dilda et al., 2011; Rosenberger et al., 2012; Lawless et al., 2013; Li et al., 2018). The fact that our results show bta-mir-193a targeting CD14, ITGAM, TLR-2, TLR-4, and TNF-α strongly supports its roles in transcriptional activation of proinflammatory response and innate immune gene expression during bovine trypanosomosis. This provides compelling evidence that this miRNA could serve as a significant biomarker in disease diagnosis and treatment. Many *in vitro* studies have elucidated the significant roles of miRNAs in regulating bovine immune responses during disease conditions (Dilda et al., 2011; Lawless et al., 2013; Wang et al., 2017; Ammah et al., 2018). The fact that TLR-2 and TLR-4 are part of bta-miR-193a target suggests its involvement in Toll-like receptor signaling during host response to trypanosomosis infection.

CONCLUSION

Our study provides computational evidence that certain miRNAs are conserved within the bovine genome and may be associated with regulation of immune response during bovine trypanosomosis. We propose that miRNAs targeting more genes will play greater roles in immune regulation and as such exert significant impact on disease phenotype. We also show that the CDS region has abundant miRNA binding site for all genes in this study demonstrating the importance of this region and requiring further elucidation in bovine disease studies. Additionally, we demonstrate that miRNA that co-target 3′UTR and CDS are important and capable of impacting target gene expressions in a complementary manner. Finally, our study identified some microRNAs including bta-mir-193a, bta-mir-2460, bta-mir-2349, and bta-mir-2888 amongst others, which might be important regulatory markers for diagnosis of bovine trypanosomosis as well as treatment and drug targets.

DATA AVAILABILITY

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

AUTHOR CONTRIBUTIONS

OM and MH performed the experiment. OM, MH, AH, and BT analyzed the data. OM, AH, and BT wrote the manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02010/full#supplementary-material

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Trypanosoma brucei Secreted Aromatic Ketoacids Activate the Nrf2/HO-1 Pathway and Suppress Pro-inflammatory Responses in Primary Murine Glia and Macrophages

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African trypanosomes, such as *Trypanosoma brucei* (*T. brucei*), are protozoan parasites of the mammalian vasculature and central nervous system that are best known for causing fatal human sleeping sickness. As exclusively extracellular parasites, trypanosomes are subject to constant challenge from host immune defenses but they have developed very effective strategies to evade and modulate these responses to maintain an infection while simultaneously prolonging host survival. Here we investigate host parasite interactions, especially within the CNS context, which are not well-understood. We demonstrate that *T. brucei* strongly upregulates the stress response protein, Heme Oxygenase 1 (HO-1), in primary murine glia and macrophages *in vitro*. Furthermore, using a novel AHADH^{in} *T. brucei* cell line, we demonstrate that specific aromatic ketoacids secreted by bloodstream forms of *T. brucei* are potent drivers of HO-1 expression and are capable of inhibiting pro-IL1β induction in both glia and macrophages. Additionally, we found that these ketoacids significantly reduced IL-6 and TNFα production by glia, but not macrophages. Finally, we present data to support Nrf2 activation as the mechanism of action by which these ketoacids upregulate HO-1 expression and mediate their anti-inflammatory activity. This study therefore reports a novel immune evasion mechanism, whereby *T. brucei* secretes amino-acid derived metabolites for the purpose of suppressing both the host CNS and peripheral immune response, potentially via induction of the Nrf2/HO-1 pathway.

Keywords: trypanosomes, keto acids, immune suppression, macrophages, glia

INTRODUCTION

Human African Trypanosomiasis (HAT), also known as African sleeping sickness, is caused by infection with the parasite *Trypanosoma brucei (T. brucei)*. There are two species of *T. brucei* which cause HAT; *Trypanosoma brucei gambiense,* which is responsible for 98% of HAT cases and results in a chronic disease course, and *Trypanosoma brucei rhodesiense*, which causes a rare

zoonotic form of HAT with an acute disease course. *T. brucei* infection is transmitted by the tsetse fly, which alongside human and animal reservoirs complete the parasite's life cycle (1). Although the disease course of HAT can vary with *T. brucei* species, the disease is fatal in all cases unless treated. Despite recent encouraging developments, existing therapies for HAT remain strain and stage dependent. There are particular issues during the meningo-encephalitic stage with many drugs causing undesirable and often dangerous side effects or exhibiting a low therapeutic index. In addition, the emergence of drug resistance strains, and difficulties in administering intensive drug regimens in the rural and impoverished communities where the majority of HAT cases are located, all contribute to the need to develop new treatment strategies against *T. brucei* infection (2).

Although the immune system has multiple lines of defense against parasitic infections, *T. brucei* has developed mechanisms to avoid immune clearance, allowing it to persist as an exclusively extracellular parasite in the host and facilitate further transmission via the tsetse fly vector (3). The best studied immune evasion strategy employed by *T. brucei* is antigenic variation of the single variable surface glycoprotein (VSG) that covers the surface of the parasite (4). Macrophages act as one of the first lines of defense against *T. brucei* infection, with M1-type immune responses such as the production of pro-inflammatory mediators TNF-α and nitric oxide (NO) recognized as particularly important in parasitemia control [reviewed in (5)]. However, as strong immune responses pose a threat to the survival of trypanosomes and are potentially deleterious to the host, *T. brucei* acts to dampen the immune response in order to evade clearance by the immune system and promote host survival (3, 6). The second, meningo-encephalitic, stage of HAT occurs when *T. brucei* penetrates the blood brain barrier and is characterized by disturbances of the central nervous system (CNS) (2). It is unclear exactly why or how trypanosomes enter the brain, however it is known that immune activation of glial cells in the CNS occurs in response to trypanosome invasion (7–9). Despite the central contribution of the CNS invasion by *T. brucei* to the pathology and mortality of HAT, relatively little is known about how trypanosomes suppress the CNS immune response to facilitate their persistence in the brain and continued survival of the host (10).

Heme-oxygenase 1 (HO-1) is a stress-inducible enzyme which catalyzes the conversion of free heme to biliverdin and iron, with the concomitant release of carbon monoxide. Biliverdin can be further metabolized to bilirubin by biliverdin reductase. HO-1 and its products, biliverdin, bilirubin and CO, are well-known for their anti-inflammatory and anti-oxidant properties (11–15). Upregulation of HO-1 has been observed in certain parasitic infections, including *Plasmodium*, *Fasciola hepatica*, and *Leishmania chagasi* (16–18). Furthermore, expression of HO-1 has been associated with inhibition of the host immune response and parasite persistence (16–19). Interestingly, increased expression of HO-1 has also been observed in a model of *T. brucei* infection, however this has been attributed as a response to trypanosomiasis-associated anemia (3). How parasites such as *T. brucei* upregulate host HO-1 expression, and its consequences for the host immune response and survival, remains poorly understood.

It has long been recognized that trypanosomiasis is accompanied by a decrease in host circulating aromatic amino acids (tryptophan, tyrosine and phenylalanine) (20–25). This decrease occurs as a result of the constitutive uptake and subsequent transamination of aromatic amino acids by an unusual cytoplasmic aspartate aminotransferase (TbcASAT) in *T. brucei* (**Supplementary Figure 1**). This transamination reaction appears essential and results in the continuous production and excretion of aromatic ketoacids which can approach millimolar levels in circulation in infected animals (26–29). Interestingly, one of these aromatic ketoacids, indole pyruvate, derived from transamination of tryptophan, strongly suppressed LPS-induced pro-inflammatory cytokine IL-1β by macrophages (30). This result raised the possibility that trypanosomes secrete aromatic ketoacids within their hosts to lessen systemic pathologies associated with a persistent infection. However, anti-inflammatory effects for the other aromatic ketoacids, hydroxy-phenylpyruvate, and phenylpyruvate, derived from transamination of phenylalanine and tyrosine, respectively, have not been reported.

In this study, we explored this idea further and investigated the effects of aromatic ketoacids in both CNS and peripheral immune cells. We demonstrate that the trypanosome secretome strongly induces expression of the anti-inflammatory enzyme, HO-1, in glial cells. Using a novel *T. brucei* cell line which can metabolize aromatic ketoacids further to aromatic hydroxyacids, we have confirmed that trypanosome-generated aromatic ketoacids are not required for *T. brucei* growth, and that they mediate the induction of HO-1 in glia by the trypanosome secretome. Therefore, we report for the first time that the induction of HO-1 by *T. brucei* is mediated by trypanosome-derived aromatic ketoacids, and that this is achieved via Nrf2 activation. Furthermore, we demonstrate that these aromatic ketoacids are capable of inhibiting pro-inflammatory immune responses by glia and macrophages. Our study suggests a novel host-pathogen interaction whereby *T. brucei* secrete metabolites for the purposes of host immune suppression, via induction of the anti-inflammatory Nrf2/HO-1 pathway.

MATERIALS AND METHODS

Reagents

The aromatic ketoacids indole pyruvic acid, hydroxy-phenylpyruvate and phenylpyruvate were purchased from Sigma Aldrich and dissolved in DMEM to a final concentration of 2 mM before use. Ultrapure lipopolysaccharide (LPS) from *E. coli* serotype O111:B4 was purchased from Invivogen.

Mice

C57BL/6 mice were bred and housed under specific pathogen free conditions in the department of Comparative Medicine, Trinity College Dublin. All procedures were performed according to regulations and guidelines of the Trinity College Dublin Ethics Committee and under licensing of the Health Product Regulatory Authority (HPRA), Ireland.

Glia Cultures

Whole brains were obtained from <1 day old C57BL/6 mice and dissected, chopped and placed in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all Sigma Aldrich). Tissue was triturated, the suspension was filtered through a sterile mesh (40 μm) and centrifuged (800 g, 5 min, 20°C). The cell pellet was resuspended and cultured in complete DMEM at 37°C in a 5% CO_2 humidified environment for 12–14 days. After 24 h, media was replaced with complete DMEM containing granulocyte macrophage-colony stimulating factor (GM-CSF; 10 ng/ml, R&D Systems) and macrophage-colony stimulating factor (M-CSF; 20 ng/ml, R&D Systems), and replaced again every 3–4 days.

Non-adherent microglia were isolated by shaking (100 rpm, 2 h at room temperature), tapping and centrifuging (800 g, 5 min, 20°C). Remaining mixed glial cells were then removed by trypsin-EDTA digestion for 5 min, counted and plated at a concentration of 2.5×10^5 cells/ml in complete DMEM.

Bone Marrow Derived Macrophage Cultures

Primary bone marrow derived macrophages (BMDM) were obtained from the hind legs of adult C56BL/6 mice. Bone marrow from the tibiae and femurs was flushed out with complete DMEM and triturated. The cell suspension was centrifuged (300 g, 5 min, 20°C) and the cell pellet resuspended in ammonium chloride solution for 2 min to lyse red blood cells. Cells were centrifuged and resuspended in complete DMEM supplemented with 20% L929 medium containing M-CSF. Cells were seeded into petri dishes at a concentration of 5×10^5 cells/ml, 10 ml per dish, and incubated at 37°C in a 5% CO_2 humidified environment. After 3 days 1 ml of L929 medium containing M-CSF was added to each petri dish. Cells were harvested on day 6 by scraping adherent cells, which were then centrifuged (300 g, 5 min, 20°C), resuspended in complete DMEM supplemented with 10% L929 medium containing M-CSF and plated at 1×10^6 cells/ml.

Culture and Growth of *Trypanosoma brucei*

The species of *T. brucei* used in this study was the monomorphic, trypomastigote stage of *Trypanosoma brucei brucei*. The strain used was MITat 1.2, also termed Lister 427-2, that were modified to express T7 polymerase and Tet repressor elements and was maintained by selection in G418 (2.5 μg/ml) and hygromycin (5 μg/ml). Trypanosomes were cultured in sterile Hirumis' Modified Iscoves' Medium, formulation 9 (HMI-9), supplemented with 10% FCS, 180 mM $NaHCO_3$, 1 mM β-mercaptoethanol, 50 mg/l ampicillin and streptomycin, at pH 7.5. Trypanosomes were cultured at 37°C in a 5% CO_2 humidified environment. Cell growth density was maintained at a range between 1×10^5 cells/ml and 2×10^6 cells/ml.

Generation of AHADH^{in} *T. brucei*

The related trypanosomatid *T. cruzi,* which causes American trypanosomiasis, does not secrete ketoacids which are instead metabolized further to the corresponding aromatic hydroxy acids by the enzyme L-alpha-hydroxyacid dehydrogenase (AHADH) (28, 29). In order to confirm that the ketoacids secreted by *T. brucei* mediate the observed increase in HO-1 expression in mixed glia treated with *T. brucei* supernatant, a novel *T. brucei* cell line with inducible AHADH expression was created. This conditional cell line was generated using p3859, a plasmid that allows tetracycline-inducible expression of transgenes (29). The complete open reading frame of AHADH (EMBL accession number AF112259, TriTrypDB gene ID TcCLB.506937.10) was amplified from *Trypanosoma cruzi* (*T. cruzi*) genomic DNA with the primers shown in **Table 1** and subcloned into the pGEM-T vector. Positive clones were selected and sequenced. In order to remove the internal Not I restriction site present in the AHADH sequence, site-directed mutagenesis was performed on the cloned AHADH (in pGEM-T) using PCR and specific primers designed to mutate a single base of the AHADH NotI site (**Table 1**). Following DpnI digestion the amplification products were used to transfect *E. coli,* subsequently positive clones were selected and confirmed by digest/sequence analysis. The complete AHADH open reading frame was then excised from pGEM-T using a Hind III/BamH I double digest, and subcloned into Hind III/BamH I double digested p3859. Insertion of AHADH into the digested p3859 was confirmed by restriction digests and PCR. The plasmid was then subjected to Not I digestion, to allow targeting of the construct to the non-transcribed spacer in the rRNA locus, and used to transfect bloodstream forms *T. brucei*. Positive clones were selected for and maintained in complete HMI-9 supplemented with 5 μg/ml blasticidin, 2.5 μg/ml G418, and 5 μg/ml hygromycin.

AHADH Assay

AHADH was used to detect the production of the ketoacids in a NADH coupled reaction as previously described (30). The decrease in absorbance at 340 nm was used to monitor the reaction; change in absorbance correlates directly to ketoacid production. The assay was performed using Tris buffer (25 mM, pH 7.4), NaCl (50 mM), NADH (0.25 mM), and AHADH (850 U), to a final volume of 1 ml.

Viability Measurement

Viability of mixed glia and BMDM was measured by reduction of alamarBlue™ (BioRad) reagent. A volume of alamarBlue equal to 10% of the cell culture volume was added to each well and

TABLE 1 | Primer sequences.

AHADH F	5′-GCGAAGCTTATGTTTTTTGAAGGTGCATGC GCGAAGGTG-3′
AHADH R	5′-CCGGATCCTTACAATGCCAAAGACAGCGA CTCCGA-3′
AHADHNotIMuta F	5′-TCATTGCCGGAGGCCGCATGTTGG-3′
AHADHNotIMuta R	5′-CCAACATGCGGCCTCCGGCAATGAGGG-3′
NQO1 F	5′-GCTGCAGACCTGGTGATATT-3′
NQO1 R	5′-TGTAGGCAAATCCTGCTACG-3′
GSR F	5′-GGAAGCAGCCCTTCATCTTT-3′
GSR R	5′-TGGCAACTGTTCCTGAACTC-3′
β-Actin F	5′-GGACTCCTATGTGGGTGACGAGG-3′
β-Actin R	5′-GGGAGAGCATAGCCCTCGTAGAT-3′

the plate was swirled gently to mix. Cells were incubated at 37°C for 6–18 h and then absorbance was read at 570 and 600 nm. The 600 nm absorbance values were subtracted from the 570 nm values, and cellular viability was expressed as a percentage of the untreated control.

Western Blotting

For detection of HO-1 expression, mixed glia or BMDM were cultured in the presence of trypanosomes, trypanosome supernatants or ketoacids (0.25–1 mM) for 24 h. For detection of pro-IL-1β and iNOS mixed glia or BMDM were cultured in the presence of ketoacids (0.25–1 mM) for 30 min prior to stimulation with LPS (100 ng/ml) for 24 h. Cell lysates were prepared by washing cells in PBS prior to lysis in RIPA buffer (Tris 50 mM; NaCl 150 mM; SDS 0.1%; Na.Deoxycholate 0.5%; Triton X 100; all Sigma-Aldrich). For detection of Nrf2 mixed glia or BMDM were cultured in the presence of ketoacids (0.25–1 mM) for 6–24 h, then washed in PBS and lysed in Laemmli loading buffer. Samples were electrophoresed and transferred to PVDF membranes which were then blocked in 5% non-fat milk and incubated with monoclonal antibodies specific for HO-1 (Enzo Life Sciences), pro-IL-1β (R&D systems), iNOS or Nrf2 (both Cell Signaling) overnight at 4°C. Membranes were then washed in TBS-Tween and incubated with appropriate streptavidin-conjugated secondary antibody (anti-rabbit or anti-goat; both Sigma Aldrich) for 2 h at room temperature, prior to development with enhanced chemiluminescent substrate (Merck Millipore) using a BioRad ChemiDoc MP system. Subsequently, membranes were re-probed with HRP-conjugated monoclonal antibodies specific for β-actin (Sigma-Aldrich) as a loading control. Full length blots are presented in **Supplementary Figures 4–8**.

Quantitative Real Time PCR

For detection of NQO1 and GSR expression by PCR mixed glia and BMDM were cultured in the presence of ketoacids (0.5–1 mM) for 24 h. RNA was extracted using the High Pure RNA Isolation Kit (Roche) and cDNA synthesized using High Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real time PCR was carried out using iTaq Universal SYBR Green mastermix (BioRad) on a BioRad CFX96 Real-Time System. mRNA expression levels for NQO1 and GSR were quantified and were normalized to β-actin mRNA levels (primer sequences listed in **Table 1**).

ELISA

For detection of cytokines, mixed glia, or BMDM were cultured in the presence of ketoacids (0.25–1 mM) for 30 min prior to stimulation with LPS (100 ng/ml) for 24 h. Concentrations of IL-6 and TNF-α were quantified from supernatants using R&D DuoSet ELISA kits (R&D Systems) or Ready-Set-Go ELISA kits (eBioscience) as per the manufacturers' protocols.

Statistical Analysis

Statistical analysis was performed using Prism 6 software (GraphPad Software Inc.). Analysis of 3 or more data sets was performed by one-way ANOVA with Tukey's *post-hoc* test; *p*-values < 0.05 were considered significant and are denoted with asterisks in the figures.

RESULTS

Trypanosoma brucei Secreted Factors Induce HO-1 Expression and Suppress Pro-Inflammatory Cytokines in Mixed Glia

The anti-inflammatory and anti-oxidant stress-response enzyme HO-1 has been described to have immunosuppressive activity during parasitic infection (16–19). We first determined whether *T. brucei* can directly impact on HO-1 expression in primary murine mixed glia. *T. brucei* were co-cultured with mixed glia at different concentrations for 24 h, and HO-1 expression was assessed by Western blot. It was observed that mixed glia cultured with *T. brucei* strongly upregulated HO-1 expression compared to mixed glia cultured in media alone (**Figure 1A**). In order to determine whether upregulation of HO-1 is mediated by direct contact between mixed glia and trypanosomes, or by trypanosome-secreted factors, supernatants from cultures of *T. brucei* were added to mixed glia at a 2- and 5-fold dilution for 24 h and HO-1 expression was assessed by Western blot. A dose-dependent upregulation of HO-1 was observed in mixed glia treated with *T. brucei* supernatant (**Figure 1B**). HO-1 expression was also assessed using trypanosomes grown under serum free conditions in order to rule out the possibility that factors contained in FCS are driving HO-1 expression. We found that removal of 10% FCS supplement leads to lower secreted ketoacid levels (**Supplementary Figure 2A**). This is also reflected by slightly lower HO-1 levels, however there is still a clear increase in HO-1 expression in glia that were treated with the trypanosome supernatant vs. cells that were treated with media containing no trypanosome supernatant (**Supplementary Figure 2B**). Additionally, we assessed whether *T. brucei* supernatant could suppress pro-inflammatory cytokine production by mixed glia. Cells were treated with *T. brucei* supernatant for 30 min prior to stimulation with LPS for 24 h, and the concentration of IL-6 and TNFα was measured by ELISA. The *T. brucei* culture supernatant significantly reduced the production of both cytokines following LPS-stimulation (**Figure 1C**).

Trypanosoma brucei Secreted Aromatic Ketoacids Are Non-toxic and Induce HO-1 Expression in Mixed Glia and Macrophages

It has previously been reported that *T. brucei* secretes the aromatic ketoacids indole pyruvate, hydroxy-phenylpyruvate, and phenylpyruvate, and that indole pyruvate can inhibit pro-IL-1β expression in BMDM via HIF1α destabilization (25, 27, 30). Having confirmed that *T. brucei* induces HO-1 expression in mixed glia, and that induction is mediated by factors secreted by the trypanosome, we next determined whether these aromatic ketoacids can directly upregulate HO-1 expression in both mixed glia and BMDM. Viability assays were carried out to ensure that the aromatic ketoacids are non-toxic to

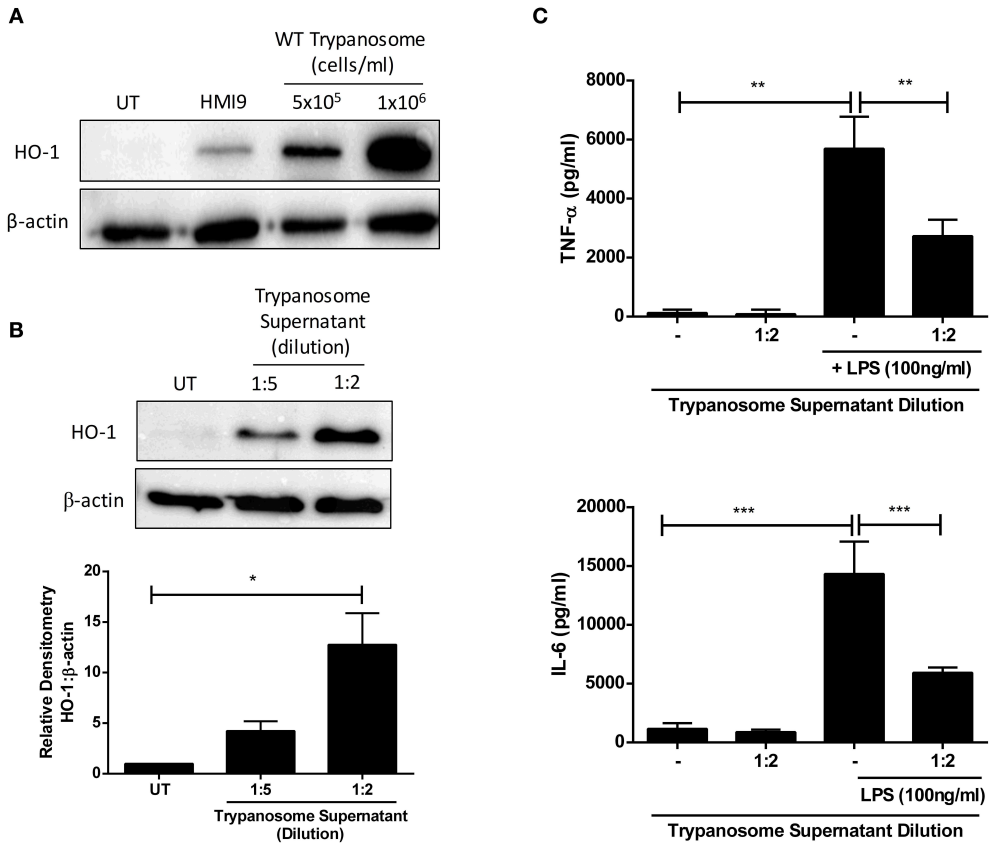

FIGURE 1 | *Trypanosoma brucei* secreted factors induce HO-1 expression & suppress pro-inflammatory cytokines in mixed glia. **(A)** Primary murine mixed glia were cultured with different concentrations of *Trypanosoma brucei* for 24 h. Expression of HO-1 was measured by Western blot. Representative blot of three independent experiments is shown. **(B)** Supernatant from *Trypanosoma brucei* cultures was added to primary murine mixed glia. Expression of HO-1 was measured after 24 h by Western blot. Representative blot of three independent experiments is shown. Densitometric analysis of 3 immunoblots was performed using ImageLab (Bio-Rad) software. Results shown are mean ± SEM of the relative expression of HO-1: β-actin from 3 independent experiments. **(C)** Supernatant from *Trypanosoma brucei* cultures was added to primary murine mixed glia for 30 min prior to stimulation with LPS (100 ng/ml). Concentrations of TNFα and IL-6 in culture supernatants was measured by ELISA after 24 h. Results shown are mean ± SD concentrations from a triplicate culture, and are representative of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Full length blots are presented in **Supplementary Figure 4**.

mixed glia and BMDM at the concentrations used in our study, which mimic typical ketoacid concentrations observed during *in vivo* trypanosomiasis (30). Mixed glia and BMDM were cultured with indole pyruvate, hydroxy-phenylpyruvate, or phenylpyruvate (0.25–1 mM) for 24 h, after which cellular viability and HO-1 expression was assessed. All three ketoacids were found to be well-tolerated and non-toxic to mixed glia (**Figure 2A**) or BMDM (**Figure 2C**). Furthermore, all three ketoacids induced a strong upregulation of HO-1 expression in both cell types (**Figures 2B,D**). This effect was dose-dependent, with indole pyruvate and hydroxy-phenylpyruvate showing greater potency compared to phenylpyruvate. Removal of serum from the media did not affect the ability of indole pyruvate or hydroxyphenylpyruvate to induce HO-1 expression in BMDM. However, only a very weak induction of HO-1 was observed by phenylpyruvate when media was replaced with serum free media, suggesting that serum components may impact on the solubility of this ketoacid (**Supplementary Figure 2C**).

The related Trypanosomatid *T. cruzi* expresses an NAD-linked aromatic α-hydroxy acid dehydrogenase (AHADH) that catalyzes the reduction of aromatic ketoacids to the corresponding hydroxyacid (**Figure 2E**) (31). Consequently, a conditional AHADH *T. brucei* cell line was generated in order to determine whether secretion of ketoacids was essential to *T. brucei* growth and to produce a bloodstream form that would secrete less ketoacids. This conditional cell line was generated using p3859, a plasmid that allows tetracycline-inducible expression of transgenes (29). Both non-induced and AHADH-induced *T. brucei* had comparable growth rates (**Figure 2F**). Significantly, both non-induced and induced AHADH(in) cells were found to have similar decreased levels of aromatic ketoacids in their culture media, which could be due to leaky expression of the AHADH(in) vector in the absence of tetracycline. In both cases there was a ∼60–70% reduction in ketoacid secretion compared to the wild type parental cell line with wild-type *T. brucei* producing

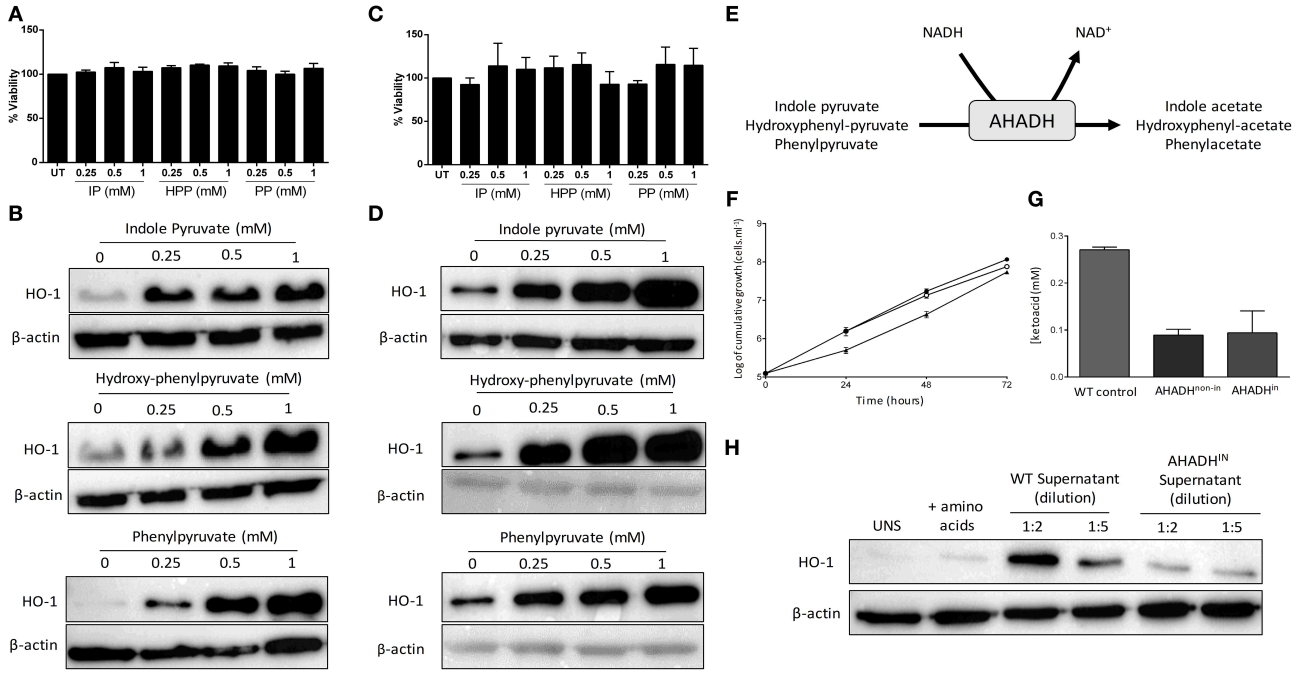

FIGURE 2 | *Trypanosoma brucei* produce and secrete aromatic ketoacids which are non-toxic and induce HO-1 expression in mixed glia and BMDM. Primary murine mixed glia and BMDM were incubated with indole pyruvate, hydroxyl-phenyl pyruvate, or phenylpyruvate (0.25–1 mM) for 24 h. **(A)** Viability of mixed glia was determined by alamarBlue reduction, and is expressed as a percentage of the untreated control. Results shown are mean ± SEM of the percentage viabilities of mixed glia from three independent experiments. **(B)** Expression of HO-1 in mixed glia was determined by Western blot. Blots shown are representative of three independent experiments. **(C)** Viability of BMDM was determined by alamarBlue reduction, and is expressed as a percentage of the untreated control. Results shown are mean ± SEM of the percentage viabilities of BMDM from four independent experiments. **(D)** Expression of HO-1 in BMDM was measured by Western blot. Blots shown are representative of two independent experiments. **(E)** Schematic depicting metabolism of aromatic ketoacids to aromatic hydroxyacids by AHADH. **(F)** Bloodstream form AHADH^{in} cells were found to grow at similar rates to wild type MITat 1.2 *T. brucei* (▲), whether induced (○) or non-induced (•). **(G)** Secreted aromatic ketoacid concentrations in HMI-9 culture media, as measured via AHADH assay after 48 h. AHADH cells were either non-induced or induced with 2 mg/ml tetracycline. Results shown are mean ± SD of triplicate measurements, and are representative of three independent experiments. **(H)** Supernatants from WT and AHADH^{in} *T. brucei* were added to mixed glia for 24 h. HO-1 expression was measured by Western blot. Representative blot of three independent experiments is shown. Full length blots are presented in **Supplementary Figure 5**.

∼2.5 times more keto acid than mutant strain (**Figure 2G**). These data show that production of aromatic ketoacids is not an essential process and that, at least in these cell lines, expression of the AHADH activity was effectively constitutive and not subject to tetracycline regulation. Having confirmed the AHADH^{in} *T. brucei* cell line exhibited reduced ketoacid secretion, and showed no growth deficiency, supernatants from wild type and AHADH^{in} *T. brucei* were added to mixed glia cultures. After 24 h, HO-1 expression was measured by Western blot. As previously observed, wild type *T. brucei* supernatant strongly upregulated HO-1 expression in mixed glia. However, the AHADH^{in} *T. brucei* supernatant displayed a dramatically reduced capacity to induce HO-1 expression in these cells. Clear differences were seen when both the wild-type and AHADH^{in} *T. brucei* supernatants were diluted 2-fold, while comparable levels of HO-1 were expressed in cells treated with the wild type *T. brucei* supernatant (diluted 1:5) and AHADH^{in} *T. brucei* supernatant (diluted 1:2) as the overall keto acid concentration in each case should be approximately equal (**Figure 2H**).

Aromatic Ketoacids Exhibit Anti-inflammatory Activity in Primary Glia and Macrophages

Having confirmed that the *T. brucei*-derived ketoacids, indole pyruvate, hydroxy-phenylpyruvate, and phenylpyruvate can upregulate HO-1 expression in mixed glia and BMDM, and given that HO-1 has well-established anti-inflammatory properties, we sought to determine if the ketoacids themselves can serve as anti-inflammatory molecules. It has previously been reported that indole pyruvate inhibits production of the highly pro-inflammatory cytokine, IL-1β, by macrophages (30), therefore we investigated whether the other ketoacids hydroxy-phenylpyruvate and phenylpyruvate had similar activity, and whether this effect would be observed in mixed glia as well as macrophages. Microglia were also included in this experiment given their macrophage-like role in the brain. Mixed glia, microglia and BMDM were treated with ketoacids for 30 min prior to stimulation with LPS. After 24 h the expression of the pro-form of IL-1β, pro-IL-1β, was detected by Western blot. Both indole pyruvate and hydroxy-phenylpyruvate

strongly inhibited pro-IL-1β expression by mixed glia at all concentrations tested, while phenylpyruvate reduced pro-IL-1β only at higher concentrations (**Figure 3A**). Similarly, indole pyruvate and hydroxy-phenylpyruvate treatment abrogated pro-IL-1β expression in microglia and BMDM, while phenylpyruvate had no effect (**Figures 3B,C**).

Inducible nitric oxide synthase (iNOS) catalyzes the production of NO from L-arginine, and contributes to pro-inflammatory responses in glia and BMDM (31, 32). In order to examine whether ketoacids could modulate iNOS expression, BMDM, and mixed glia were pre-treated with ketoacids and stimulated with LPS as before. After 24 h iNOS expression was measured by western blot. Expression of iNOS was below the limit of detection in mixed glia however, the enzyme was upregulated in LPS-stimulated BMDM (**Figure 3D**). As was the case with pro-IL-1β, iNOS expression was abrogated in the cells upon treatment with the ketoacids.

Having observed reduced expression of pro-IL-1β upon ketoacid treatment, we next examined whether they were also capable of reducing expression of the pro-inflammatory cytokines, IL-6 and TNFα, in primary glia and BMDM. Mixed glia and BMDM were pre-treated with ketoacids and stimulated with LPS as before and the concentration of IL-6 and TNFα in culture supernatants was measured by ELISA. Both indole pyruvate and hydroxy-phenylpyruvate significantly reduced IL-6 and TNFα production by mixed glia, and this inhibition was dose dependent. Phenylpyruvate did not reduce either IL-6 or TNFα production by mixed glia (**Figure 4A**). Conversely, none of the ketoacids tested had any effect on IL-6 or TNFα production by BMDM (**Figure 4B**). Attempts were made to make a direct link between HO-1 induction and cytokine inhibition by the ketoacids, however this proved technically difficult given the challenge of simultaneously inducing and inhibiting the HO-1 protein, as has been previously reported (33). High doses of HO-1 inhibitors and siRNA were employed in an attempt to counter the potent induction of HO-1 by the ketoacids, but this unfortunately reduced the viability of our cells, particularly when used in combination with indole pyruvate and phenylpyruvate treatment. We did however observe that inhibition of HO-1 partially restored LPS-induced pro-IL-1β expression in hydroxyphenylpyruvate treated BMDM (**Supplementary Figure 3**). Further work in HO-1 deficient cells is required to confirm the direct link between HO-1 induction and cytokine inhibition by the ketoacids.

Ketoacids Activate Nrf2 in Mixed Glia and BMDM

Finally, to elucidate the mechanism by which *T. brucei* derived ketoacids upregulate HO-1 in mixed glia and BMDM, we next investigated whether the ketoacids activate the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) which regulates the expression of a number of anti-oxidant genes, and is the primary regulator of HO-1. Under steady-state conditions Nrf2 is bound to Keap1 and targeted for degradation; however, during oxidative stress Nrf2 is released from Keap1 and can migrate to the nucleus (34). To measure Nrf2 accumulation, mixed glia and BMDM were treated with ketoacids for up to 24 h, and Nrf2 was detected by Western blot. Both indole pyruvate and hydroxy-phenylpyruvate dose-dependently increased Nrf2 accumulation in mixed glia (**Figure 5A**) and BMDM (**Figure 5B**). Nrf2 expression was not detected with phenylpyruvate in either cell type. To confirm activation of the Nrf2 pathway, we measured expression of additional Nrf2-regulated genes following ketoacid treatment. Mixed glia and BMDM were treated with ketoacids for 24 h, after which the expression of NAD(P)H dehydrogenase (quinone 1) (NQO-1) and glutathione reductase (GSR), was measured by RT-PCR. In mixed glia, indole pyruvate and hydroxy-phenylpyruvate both significantly increased expression of NQO-1, and hydroxy-phenylpyruvate also showed a trend toward increased GSR expression. Phenylpyruvate did not upregulate expression of either gene (**Figure 5C**). Similarly, in BMDM, both indole pyruvate and hydroxy-phenylpyruvate significantly upregulated NQO-1 expression, while only indole pyruvate showed a trend toward increased GSR expression, and phenylpyruvate again had no effect on either gene (**Figure 5D**).

DISCUSSION

HAT is a chronic parasitic disease, associated with considerable morbidity and mortality, that is caused by African trypanosomes. The challenge facing trypanosomes is to maintain a persistent infection while simultaneously prolonging host survival in order to potentiate parasite transmission to the tsetse fly vector to ensure completion of the life cycle. This challenge is of particular relevance for trypansomes which are exclusively extracellular and, unusually for a pathogen, can invade the CNS. Here we show that *T. brucei* can upregulate expression of the anti-inflammatory stress-response enzyme HO-1 within host CNS and peripheral immune cells. Significantly, all three aromatic ketoacids secreted by *T. brucei*, indole pyruvate, hydroxy-phenylpyruvate, and phenylpyruvate, can mediate this induction of HO-1. Therefore, our results not only describe a previously unknown function for the production of aromatic ketoacids by *T. brucei*, but also a novel host-pathogen interaction whereby *T. brucei* has evolved to secrete metabolites designed to promote an overall anti-inflammatory response in the host.

Although upregulation of HO-1 expression has been observed in other parasitic infections (16–18), relatively little is known about the role of HO-1 in trypanosomiasis. Expression of HO-1 has been observed in a murine model of *T. cruzi* infection by Gutierrez et al. who also examined the effect of HO-1 activity on the host immune response and survival. In their study, treatment with a HO-1 inhibitor exacerbated inflammation and limited host survival, while conversely a HO-1 inducer reduced production of pro-inflammatory cytokines and promoted anti-inflammatory responses, including production of IL-10 and induction of Tregs (19). In the present study, we observed a strong upregulation of HO-1 in glial cells cultured with *T. brucei*. In agreement with this observation, Stijlemans et al. have reported increased expression of HO-1 within a murine model of *T. brucei* infection

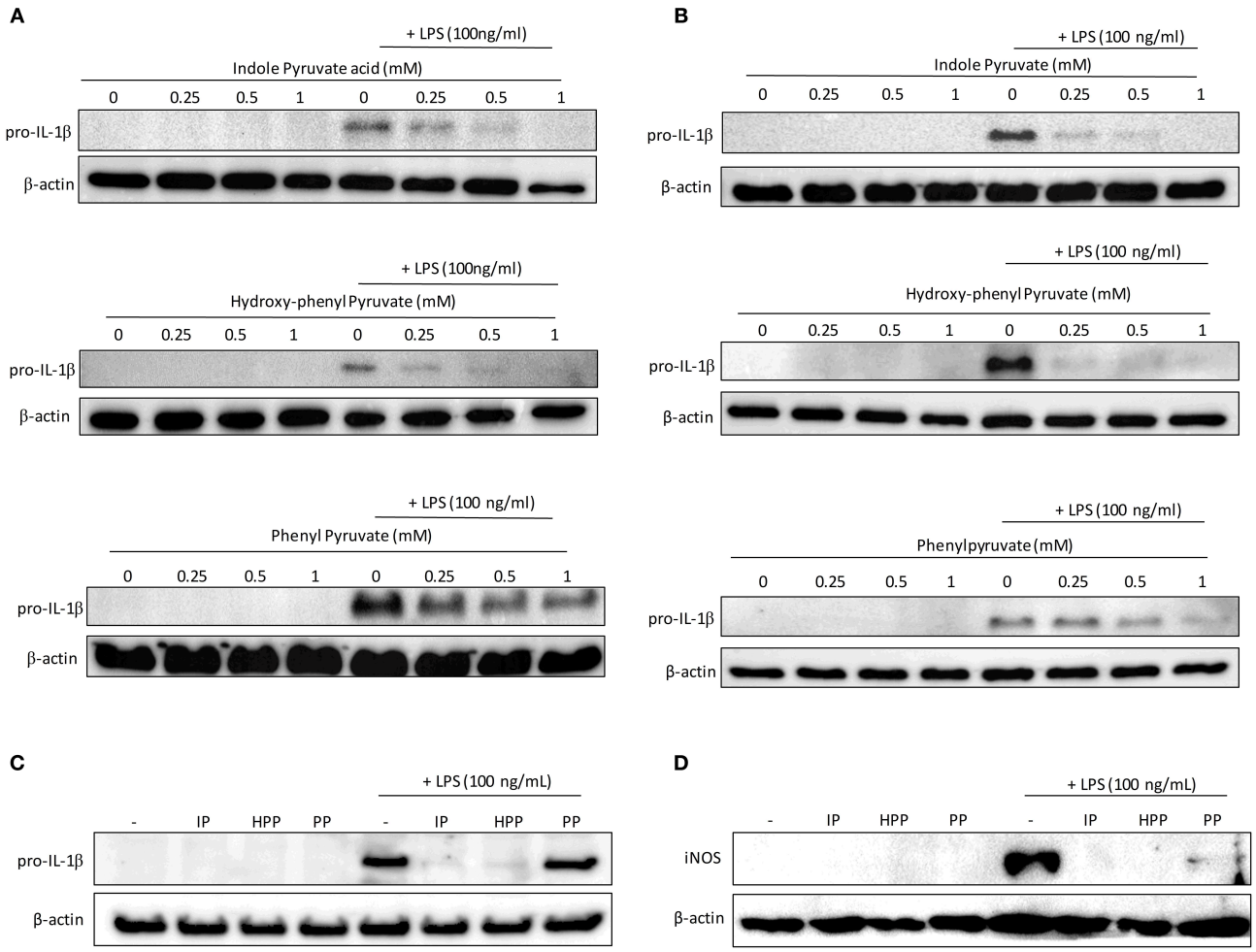

FIGURE 3 | Ketoacids inhibit pro-IL-1β expression in LPS-stimulated mixed glia, microglia, and BMDM. Primary murine mixed glia, microglia and BMDM were treated with indole pyruvate (IP), hydroxy-phenylpyruvate (HPP), or phenylpyruvate (PP) (0.25–1 mM) for 30 min prior to stimulation with LPS (100 ng/ml) for 24 h. Expression of pro-IL-1β in **(A)** mixed glia, **(B)** microglia, and **(C)** BMDM was measured by Western blot. Blots shown are representative of two to three independent experiments. Expression of iNOS in **(D)** BMDM was measured by Western blot. Blots shown are representative of three independent experiments. Full length blots are presented in **Supplementary Figure 6**.

(5). Additionally, they have reasoned this upregulation of HO-1 as a response to anemia resulting from the accumulation of iron by trypanosomes (35). As it is currently unknown whether *T. brucei* themselves express the cellular machinery necessary to extract iron from heme-containing proteins (36), it is tempting to speculate that they have evolved to upregulate HO-1 in their host for the purposes of both increasing their supply of iron and creating a trypanotolerant immune environment.

Despite reports that increased HO-1 expression is a feature of parasitic infections, it is unknown how parasites including trypanosomes achieve this upregulation of HO-1 in their host. To clarify how *T. brucei* mediates its upregulation of HO-1, we investigated whether a factor secreted by the trypanosome may be responsible for this effect. We found that treatment of mixed glia with *T. brucei* supernatant produced a similar upregulation of HO-1 expression, and additionally suppressed pro-inflammatory cytokine production. This observation is in agreement with a previous study by Garzón et al. who reported that treatment of murine BMDC with *T. b. gamiense* secretome effectively limited DC maturation and pro-inflammatory functions (37). However, the specific factors present in the *T. b. gamiense* secretome which mediate these anti-inflammatory effects have not been identified. It is known that *T. brucei* infections are associated with disturbed aromatic amino acid profiles, owing to the metabolism of aromatic amino acids into aromatic ketoacids by *T. brucei* (25–27). To date, however, there has been limited research identifying the purpose of these ketoacids. It has recently been reported that one of these ketoacids, indole pyruvate, has anti-inflammatory effects in BMDM (30). We therefore hypothesized that ketoacids could be factors secreted by *T. brucei* which mediate our observed anti-inflammatory effects, via induction of HO-1. To test this hypothesis we treated both

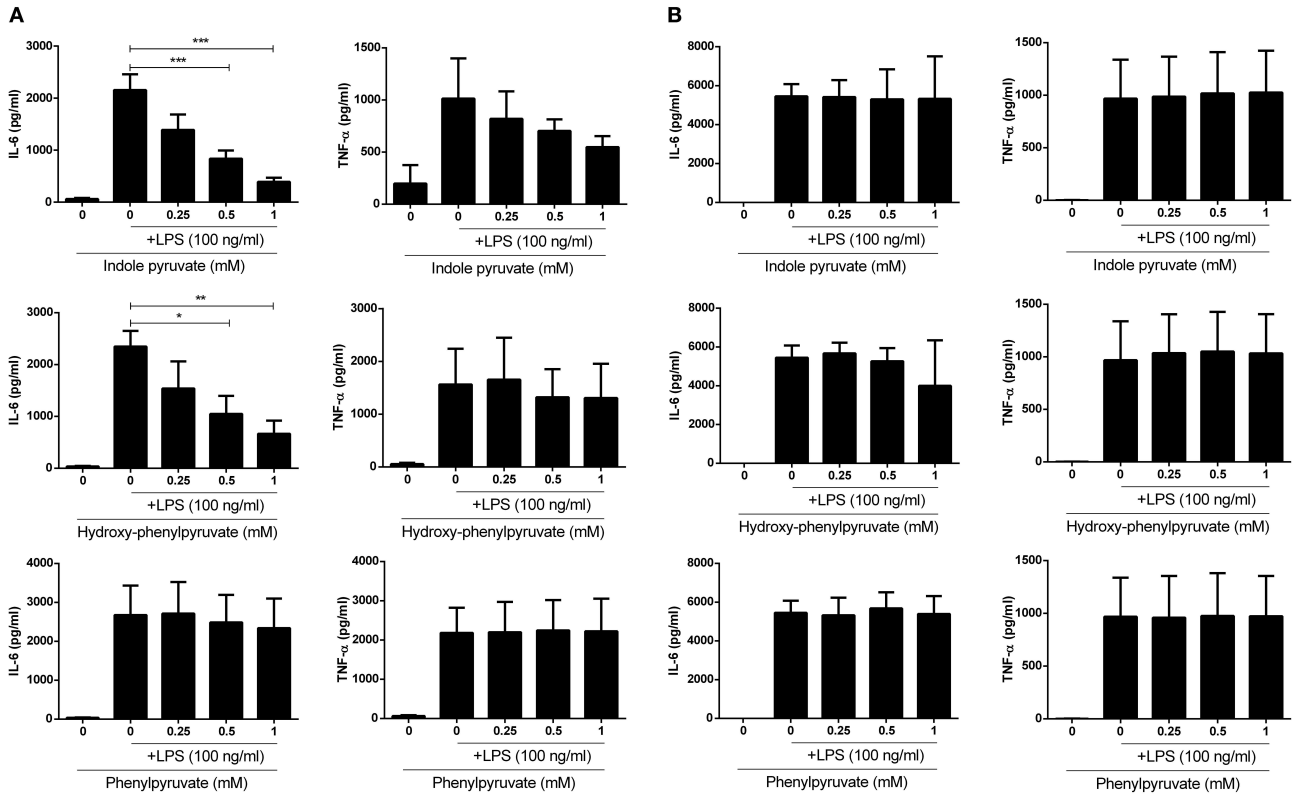

FIGURE 4 | Indole pyruvate and hydroxyl-phenyl pyruvate inhibit pro-inflammatory cytokine production in LPS-stimulated microglia but not BMDM. Primary murine microglia and BMDM were treated with indole pyruvate, hydroxy-phenylpyruvate, or phenylpyruvate (0.25–1 mM) for 30 min prior to stimulation with LPS (100 ng/ml) for 24 h. Concentrations of TNFα and IL-6 in **(A)** mixed glia and **(B)** BMDM supernatants was measured by ELISA. Results shown are mean ± SEM concentrations of IL-6 and TNFα from three to six independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

BMDM and glia with three different ketoacids, indole pyruvate, hydroxyl-phenyl pyruvate, and phenylpyruvate, and found that all three were capable of strongly upregulating HO-1 expression. Furthermore, we created a novel *T. brucei* cell line which possesses inducible expression of the ADADH enzyme, which catalyzes the conversion of ketoacids to hydroxyacids. Using this ADADH^{in} *T. brucei* we were able to confirm that aromatic ketoacids are mediators of HO-1 upregulation within the *T. brucei* secretome. To our knowledge, the present study is the first to identify *T. brucei* derived ketoacids as HO-1 inducers. Further study using cASAT deficient trypansosomes will determine if other *T. brucei* secreted factors are also capable of inducing this stress response pathway. In addition, future studies will determine the precise concentration of the individual keto acids produced by the parasite, given that the ADADH enzyme assay which is used to measure overall ketoacid production does not distinguish between indole pyruvate, phenylpyruvate, and hydroxyphenyl- pyruvate.

While there have been previous studies investigating evasion by *T. brucei* of the peripheral immune system, studies examining the effects of *T. brucei* on the CNS immune response have been scarce, presumably due to the difficulties inherent in accessing CNS cells. Nonetheless, this presents a significant obstruction to our understanding of HAT, especially considering that invasion of the CNS by *T. brucei* is an important clinical event in the course of this disease. In order to further our knowledge of immune evasion by *T. brucei* in the CNS, we elected to use primary murine glial cells, as well as BMDM, in the present study. Following our earlier experiments demonstrating that *T. brucei* supernatant inhibits pro-inflammatory cytokine production by glial cells, and our identification of aromatic ketoacids as *T. brucei* derived factors responsible for the upregulation of HO-1, we performed further experiments to test whether these ketoacids also possess anti-inflammatory activity in glia and BMDM. We found that all three ketoacids produced anti-inflammatory effects, with indole pyruvate and hydroxy-phenylpyruvate displaying greater activity over phenylpyruvate. In both glia and BMDM we observed a marked reduction in pro-IL-1β, along with a reduction in iNOS expression in BMDM, in response to LPS stimulation with ketoacid treatment. Interestingly, while we observed a significant reduction in IL-6 and TNFα production by glia treated with indole pyruvate and hydroxy-phenylpyruvate, no reduction of these cytokines was seen in BMDM treated with either ketoacid. This result is in agreement with a previous report that indole pyruvate reduced IL-1β, but not IL-6 or TNFα, in murine macrophages (30). This divergence in responses

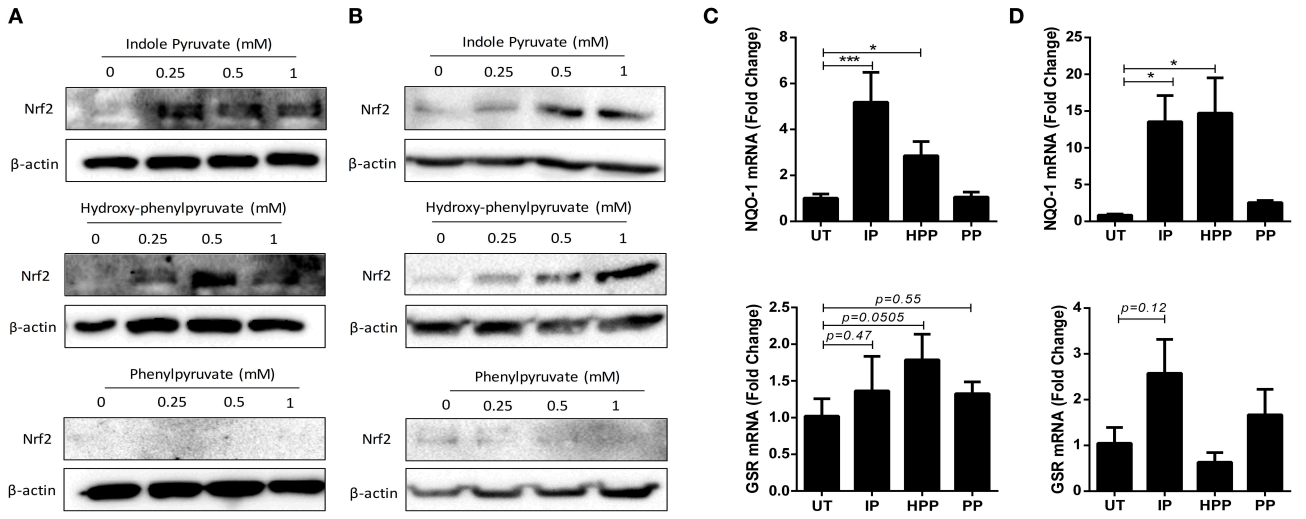

FIGURE 5 | Ketoacids induce Nrf2 expression and upregulate Nrf-2 dependent genes in mixed glia and BMDM. **(A)** Primary murine mixed glia were treated with indole pyruvate, hydroxy-phenylpyruvate, or phenylpyruvate (0.25–1 mM) for 24 h. Expression of Nrf2 was measured by Western blot. Blots shown are representative of three independent experiments. **(B)** BMDM were treated with indole pyruvate, hydroxy-phenylpyruvate or phenylpyruvate (0.25–1 mM) for 24 h. Expression of Nrf2 was measured by Western blot. Blots shown are representative of three independent experiments. **(C)** Primary murine mixed glia were treated with indole pyruvate (IP), hydroxy-phenylpyruvate (HPP), or phenylpyruvate (PP) (0.5 mM) for 24 h. mRNA expression of NQO-1 and GSR was measured by RT-PCR. **(D)** BMDM were treated with indole pyruvate, hydroxy-phenylpyruvate, or phenylpyruvate (1 mM) for 24 h. mRNA expression of NQO-1 and GSR was measured by RT-PCR. Results shown are mean ± SEM fold expression of NQO-1 and GSR from three independent experiments. $^{***}p < 0.001$, $^{*}p < 0.05$. Full length blots are presented in **Supplementary Figure 7**.

between glia and BMDM is very interesting and may reflect the greater need to avoid immune activation or inflammation within the CNS during trypanosomiasis in order to prolong host survival and, therefore, persistence of the parasite. Indeed, in a model of *T. brucei* infection, treatment with antagonists of IL-1β and TNFα attenuated neurodegeneration, highlighting the significant role these pro-inflammatory cytokines play in trypanosomiasis-associated neuropathology (38). Furthermore, HO-1 upregulation has been shown to be protective during neurological insults. For example, induction of HO-1 has been reported to improve outcomes in models of autoimmune neuroinflammation (39) and stroke (40). Further research is required to identify the mechanism(s) underlying the difference in responses to *T. brucei* derived ketoacids between glia and macrophages. It will also be of interest to determine if levels of ketoacids in the CNS are comparable to those seen in the circulation during trypanosomiasis.

Finally, to further understand how the ketoacids upregulate HO-1 expression in glia and BMDM we investigated upstream engagement of the transcription factor Nrf2. Nrf2 is typically activated under conditions of oxidative stress, and promotes the expression of anti-oxidant proteins including HO-1, NQO-1, and GSR. We found that both indole pyruvate and hydroxy-phenylpyruvate treatment stabilized Nrf2 expression, and increased expression of NQO-1 and GSR mRNA, in both glia and BMDM. We were unable to detect increased expression of Nrf2, NQO-1, or GSR with phenylpyruvate treatment, which may reflect its less potent induction of HO-1 and anti-inflammatory activity compared to the other ketoacids. Activation of the Nrf2 pathway has been described as an important regulator of the stress response in parasitic infections, which protects the host from deleterious immune activation, at the expense of persistence of the infection (41). The role of Nrf2/HO-1 in *T.cruzi* infection has been highlighted by Paiva et al. who report that the suppression of oxidative stress by this pathway promotes parasitemia (42). Interestingly, Aoki *et al.* have reported that the aromatic ketoacids protect against UV-B induced skin damage, by reducing cytotoxicity and the production of IL-1β and IL-6 (43). Both Nrf2 and HO-1 have been reported as protective against the oxidative damage caused by UV radiation (44–46). Further work is required to identify what role the Nrf2/HO-1 pathway plays in *T. brucei* infection, and how ketoacids produced by *T. brucei* activate Nrf2.

In conclusion, we have presented convincing data to support a hitherto-unidentified role for the *T. brucei* derived aromatic ketoacids in host immune evasion, which involves activation of the stress response Nrf2/HO-1 pathway. This immunosuppressive mechanism appears to be effective vs. immune responses in both the CNS and periphery, however, the effects produced are cell-type dependent. It is striking that aromatic ketoacids secreted by bloodstream forms of *T. brucei* can promote an anti-inflammatory response while simultaneously suppressing production of pro-inflammatory cytokines by immune cells. Further research is required to confirm the overall anti-inflammatory effects of these ketoacids *in vivo* and their link with activation of Nrf2/HO-1. It is hoped that future research in this area can yield new tools to treat HAT,

and perhaps utilize aromatic ketoacids as treatments for other inflammatory diseases.

AUTHOR SUMMARY

African trypanosomes, such as *Trypanosoma brucei* (*T. brucei*) are protozoan parasites best known for causing Human African Trypanosomiasis (HAT) which, if untreated, is fatal. Current therapies have limitations, while vaccination or prophylactic intervention is not possible. Trypanosomes are exclusively extracellular and evade the immune defenses by constantly changing the single protein that covers their surface. This mechanism results in characteristic waves of parasitemia as the parasite numbers rise and then fall following host antibody mediated lysis only for a new antigenic wave to emerge. The parasite load is huge and their clearance repeatedly releases large amounts of parasite material systemically with the potential to drive harmful host innate and inflammatory responses. Here we report a previously unknown mechanism by which *T. brucei* can suppress these responses. We show that aromatic ketoacids, derived from amino acids that are constitutively secreted by the parasite into the host, are potent inducers of the anti-inflammatory and stress response enzyme, Heme Oxygenase 1 (HO-1). We also show that these same ketoacids reduce inflammation in both peripheral and CNS immune cells. Our study provides new insight into how *T. brucei* manipulates the host immune system and persists in the host. Increased understanding of how ketoacids upregulate HO-1, and what their role is during HAT, may pave the way for the development of new treatments for HAT and other inflammatory diseases.

DATA AVAILABILITY

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

ETHICS STATEMENT

The animal study was reviewed and approved by all procedures were performed according the regulation, guideline, and under licensing of the Health Product Regulatory Authority (HPRA), Ireland.

AUTHOR CONTRIBUTIONS

NC, DW, PB, DN, and AD conceptualized and designed experiments. NC, DW, HF, and CC performed experiments. PB and DN designed and created the AHADH^{in} *T. brucei* cell line. NC, HF, DN, and AD wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02137/full#supplementary-material

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Naturally Acquired Antibody Response to Malaria Transmission Blocking Vaccine Candidate Pvs230 Domain 1

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Plasmodium vivax malaria incidence has increased in Latin America and Asia and is responsible for nearly 74.1% of malaria cases in Latin America. Immune responses to *P. vivax* are less well characterized than those to *P. falciparum,* partly because *P. vivax* is more difficult to cultivate in the laboratory. While antibodies are known to play an important role in *P. vivax* disease control, few studies have evaluated responses to *P. vivax* sexual stage antigens. We collected sera or plasma samples from *P. vivax*-infected subjects from Brazil ($n = 70$) and Cambodia ($n = 79$) to assess antibody responses to domain 1 of the gametocyte/gamete stage protein Pvs230 (Pvs230D1M). We found that 27.1% (19/70) and 26.6% (21/79) of subjects from Brazil and Cambodia, respectively, presented with detectable antibody responses to Pvs230D1M antigen. The most frequent subclasses elicited in response to Pvs230D1M were IgG1 and IgG3. Although age did not correlate significantly with Pvs230D1M antibody levels overall, we observed significant differences between age strata. Hemoglobin concentration inversely correlated with Pvs230D1M antibody levels in Brazil, but not in Cambodia. Additionally, we analyzed the antibody response against Pfs230D1M, the *P. falciparum* ortholog of Pvs230D1M. We detected antibodies to Pfs230D1M in 7.2 and 16.5% of Brazilian and Cambodian *P. vivax*-infected subjects. Depletion of Pvs230D1M IgG did not impair the response to Pfs230D1M, suggesting pre-exposure to *P. falciparum*, or co-infection. We also analyzed IgG responses to sporozoite protein PvCSP (11.4 and 41.8% in Brazil and Cambodia, respectively) and to merozoite protein PvDBP-RII (67.1 and 48.1% in Brazil and Cambodia, respectively), whose titers also inversely correlated with hemoglobin concentration only in Brazil. These data establish patterns of seroreactivity to sexual stage Pvs230D1M and show similar antibody responses among *P. vivax*-infected subjects from regions of differing transmission intensity in Brazil and Cambodia.

Keywords: malaria, *Plasmodium vivax*, Pvs230, transmission-blocking vaccine, seroreactivity

INTRODUCTION

Malaria is a vector-borne infectious disease caused by the *Plasmodium* protozoan parasite. Over 200 million people suffer malaria episodes every year, primarily in tropical low-income settings, and pregnant women and children are particularly vulnerable to severe disease (1). Malaria eradication is a global priority, and an efficacious vaccine could strengthen current control efforts and enable elimination strategies. Vaccine development depends on the understanding of protective immunity, and it is fundamental to characterize immune responses to infection in a natural setting. While much research has focused on *P. falciparum*, the species causing most morbidity and mortality, immune responses to *P. vivax* infection are less well studied.

In 2017, Brazil reported an increase in malaria incidence rate that contributed to 25% of malaria cases in all of Latin America, the majority of which (74.1%) were caused by *P. vivax* infection (1). But not only the Americas are affected by vivax malaria. Cambodia, in Asia, is particularly affected by malaria, reporting a 98% increase in clinical cases between 2016 and 2017 (1). Neither Cambodia nor Brazil are expected to meet the goal of 40% malaria reduction by 2020, thus, both countries require additional strategies to control and prevent malaria infection and transmission. Importantly, vivax malaria is a global issue (2) and an increase in the number of *P. vivax* cases has been recently reported in Africa (3–6).

Prevention tools that target the sexual stages of parasites may be critical to reduce disease incidence in locations where transmission rates are increasing. Transmission to the next vulnerable human can be halted by disrupting the development of the sexual stage parasite in the mosquito, the basis for the development of transmission-blocking vaccines (TBV) (7). Naturally acquired immunity to *P. falciparum* TBV candidates is well characterized (8–10) and TBVs for *P. falciparum* are currently in pre-clinical and clinical trials (11–14). However, *P. vivax* TBV candidates are less advanced. To date, only Pvs25, a post-fertilization antigen present on the surface of *Plasmodium* zygotes and ookinetes, has been evaluated as a human vaccine targeting *P. vivax* sexual stages (15, 16). Although Pvs25 immunization has shown promising results in mice, achieving durable anti-Pvs25 antibody responses remains challenging and no boosting effect of natural exposure is expected, thus multiple vaccinations may be required. We hypothesize that the development of a vaccine able to target a pre-fertilization antigen may benefit from boosting during natural infections and thereby reduce transmission more effectively. Pvs230 (the ortholog of the *P. falciparum* Pfs230) is a pre-fertilization gametocyte/gamete antigen in *P. vivax* parasites with a low level of polymorphism worldwide (17), making it a promising target for TBV strategies in Asia and Latin America. Studies have explored Pvs230 TBV candidacy by assessing mouse antisera raised against four domains of the Pvs230 protein (18), but prevalence of anti-Pvs230 antibodies during naturally acquired infection in humans has never been assessed.

Here, we evaluated seroprevalence to the first domain of the sexual stage antigen Pvs230 (Pvs230D1M) in *P. vivax*-infected subjects in malaria-endemic areas of Brazil and Cambodia. Our results can inform future strategies to develop Pvs230D1M as a transmission-blocking vaccine.

METHODS

Ethical Approvals, Study Sites, Patients, and Sample Collections

Prior approval of the clinical study protocols was obtained from the Centro de Pesquisa em Medicina Tropical (CAAEs: 0008.0.046.000-11, 0449.0.203.000-09) and the Ethics Committee of the Federal University of Minas Gerais (CAAE: 27466214.0.0000.5149) in Brazil, and the National Ethics Committee for Human Research (ClinicalTrials.gov Identifier: NCT00663546) in Cambodia, and by the Institutional Review Board, NIAID, NIH. Written informed consent was obtained from each participant.

Seventy serum samples were obtained from adults from Rondônia state, Brazil during 2011 and 2014, and 79 plasma samples were obtained from children and adults from Pursat province, Cambodia in 2010 (**Table 1**, **Figure 1**) when they presented with acute *P. vivax* infection. Presence of *P. vivax* parasites was diagnosed by microscopy and absence of *P. falciparum* parasites was also established; gametocytes were not separately documented by microscopy and are hence not available for analyses. Sera (Brazil) or plasma (Cambodia) were frozen and transported to NIH in Rockville, USA, for further analysis. Additional information on patients from this study is presented in **Table S1**.

Pvs230D1M, Pfs230D1M, PvDBP-RII, and PvCSP ELISA

Antibody responses against Pfs230D1M, Pvs230D1M, PvDBP-RII (*P. vivax* Duffy Binding Protein Region II) and PvCSP *(P. vivax* Circumsporozoite Protein) recombinant antigens were determined by enzyme-linked immunosorbent assay (ELISA). Pfs230D1M was expressed in *Pichia pastoris* as previously described (19). Details for the production and purity of Pvs230D1M (Sal-1, NCBI reference sequence XP_001613020.1)

TABLE 1 | Main demographic characteristics of study participants in Brazil and Cambodia.

		Brazil	Cambodia
Sex*	Male (%)	53 (76.8%)	61 (77.2%)
	Female (%)	16 (23.2%)	18 (22.8%)
Age (years)**	1–9 years	0 (0%)	8 (10.1%)
	10–19 years	0 (0%)	26 (32.9%)
	>20 years	67 (100.0%)	45 (57.0%)

*Samples were predominantly from male subjects. In Brazil, only samples from adults were collected. Additional demographic information is provided in **Table S1**.*

**Information not available for 1 Brazilian subject.*

***Information not available for 3 Brazilian subjects.*

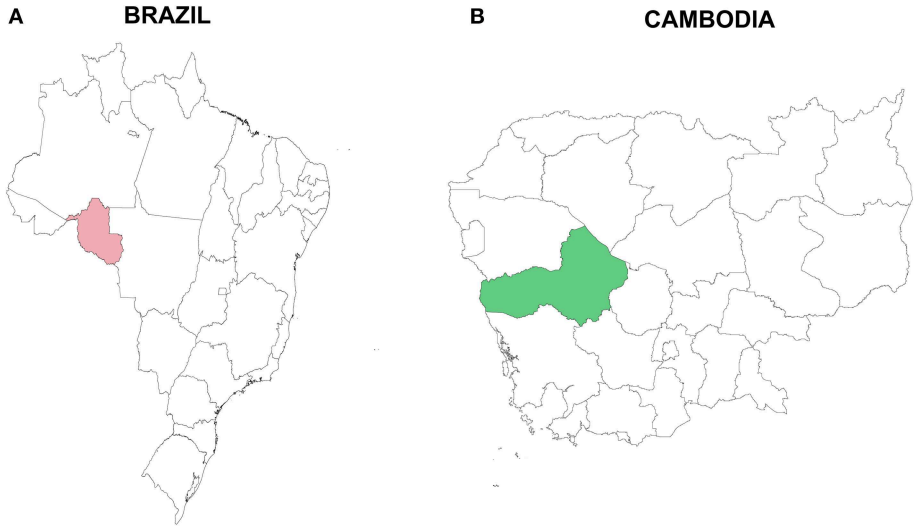

FIGURE 1 | Study sites in **(A)** Rondônia state, Brazil, South America (pink), and **(B)** Pursat province, Cambodia, Asia (green). Samples were collected in Brazil in 2011 and 2014 and in Cambodia during 2010.

and PvCSP (CSP31VK210, NCBI reference KT588189.1), which were also produced in *P. pastoris*, will be reported elsewhere [manuscript in preparation]. PvDBP-RII was expressed in *E. coli* BL-21 cells and refolded as previously described (20–23).

Immulon® 4HBX plates were coated with 1 μg/mL of recombinant antigens, then incubated overnight at 4°C. Coated plates were blocked with 320 μL of buffer containing 5% skim milk in Tris-buffered saline (TBS) for 2 h at room temperature (RT), and washed four times with 1X Tween-TBS. After establishing minimum serum dilutions to detect reactivity against individual antigens in pilot studies, plasma, or serum samples (diluted 1:10 for Pvs230D1M, 1:100 for Pfs230D1M, 1:50 for PvDBP-RII, and 1:250 for PvCSP in blocking buffer) were added to antigen-coated wells in duplicate and incubated for 2 h at RT. Plates were washed and incubated with 100 μL anti-human IgG (1:2000 dilution; SeraCare: KPL) for 2 h at RT. The plates were washed and subsequently incubated in the dark for 30 min at RT with a colorimetric substrate (p-nitrophenyl phosphate; Sigma). Absorbances (405 and 650 nm) were measured using SoftMax Pro7 ELISA reader (Molecular Devices). The cut-off to define positivity was based on the average optical density (OD) from 36 non-immune serum samples from USA donors (negative controls), whose values did not differ significantly between experiments ($p>0.9$) and hence OD values for controls from different assays were combined for (**Figures 2**, **3**, **7**). The cut-off for positivity was calculated as the mean OD of negative controls plus 3 standard deviations.

Detection of Pvs230D1M IgG Subclasses by ELISA

Immulon® 4HBX plates were coated with 5 μg/mL of recombinant Pvs230D1M antigens, then incubated overnight at 4°C. Coated plates were blocked with 320 μL of buffer containing 5% skim milk in Tris-buffered saline (TBS) for 2 h at RT, and washed four times with 1X Tween-TBS. Plasma or serum samples were diluted 1:10 in blocking buffer and were added to antigen-coated wells in duplicate and incubated for 2 h at RT. IgG subclasses were detected using the following antibodies: mouse anti-human IgG1 Fc-AP, mouse anti-human IgG2 Fc-AP, mouse anti-human IgG3 Hinge-AP, and mouse anti-human IgG4 Fc-AP from Southern Biotech for 2 h at RT. All these antibodies were diluted 1:750 in blocking buffer. The plates were washed and subsequently incubated in the dark for 30 min at RT with a colorimetric substrate (p-nitrophenyl phosphate; Sigma). Absorbances (405 nm and 650 nm) were measured using SoftMax Pro7 ELISA reader (Molecular Devices). The cut-off to define positivity was based on the average optical density (OD) from 36 non-immune sera samples from USA donors (negative controls). The cut-off for positivity was calculated as the mean OD of negative controls plus 3 standard deviations. A sample was considered positive if background-adjusted OD was above the cut-off value.

Antibody Depletion and ELISA

Immulon® 4HBX plates were coated with 1 μg/ml of recombinant Pfs230D1M, Pvs230D1M or PvDBP-RII respectively, incubated overnight at 4°C, blocked for 2 h at RT, and washed. Thereafter, 100 μl of sample (diluted 1:50) were added to Pvs230D1M-coated wells and incubated for 1 h at RT. The unbound material from the Pvs230D1M coated plate was collected and transferred into another well coated with same antigen. After the third transfer, depleted antibodies were transferred to Pfs230D1M- or PvDBP-RII-coated plates and incubated for 1 h at RT, and further processing was performed as described above. IgG responses were considered cross-reactive if preincubation with Pvs230D1M resulted in reduced antibody reactivity in Pfs230D1M ELISA. If preincubation with Pvs230D1M did not reduce antibody reactivity in the Pfs230D1M

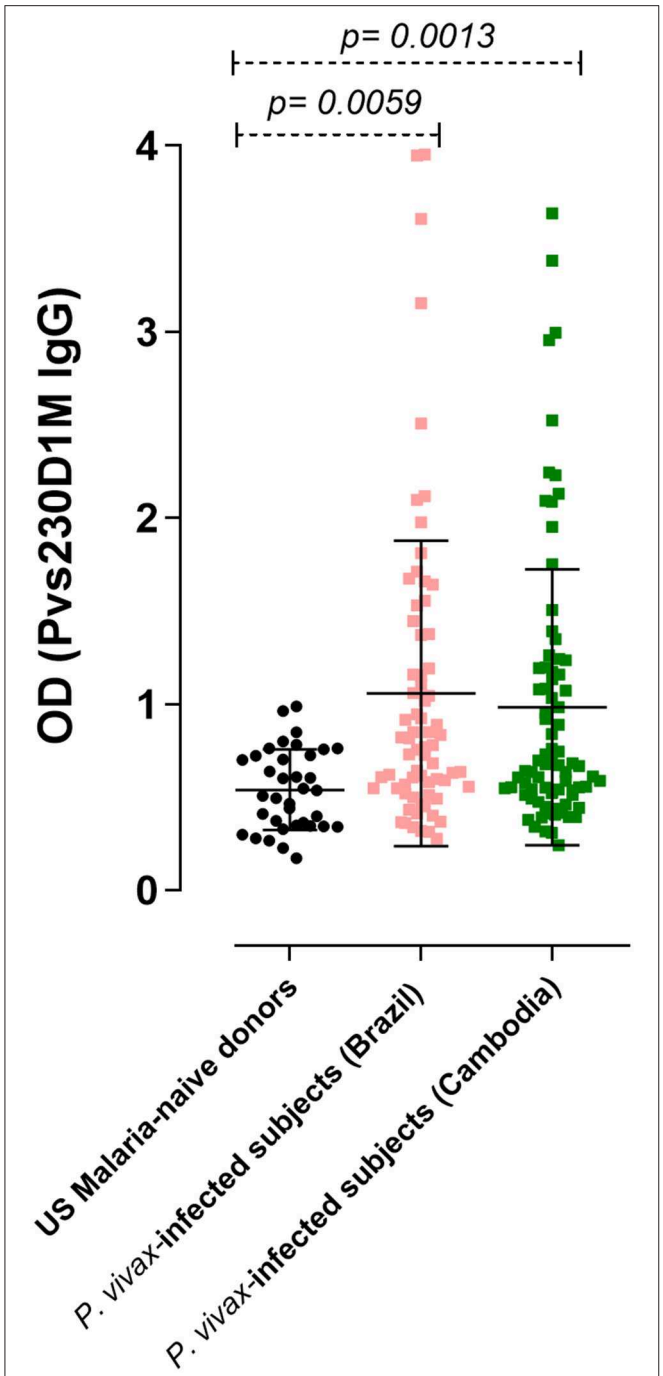

FIGURE 2 | IgG response against Pvs230D1M in *P. vivax*-infected subjects, determined by ELISA. The seroprevalence of antibodies with specificity for Pvs230D1M in Brazil was 27.1% (19/70 subjects), and in Cambodia 26.6% (21/79 subjects). Cut-off value (1.18) was calculated based on mean control + 3 standard deviations. One-Way ANOVA followed by multiple comparisons was used for this analysis and results are displayed as mean ± SD.

ELISA, the IgG response against Pfs230D1M in *P. vivax*-infected subjects was presumed to be due to *vivax*/*falciparum* co-infection or to pre-exposure to *P. falciparum*. Pvs230D1M-depleted sera were transferred to PvDBP-RII-coated plates, to confirm that the depletion or reduction found in Pvs230D1M ELISA was specific for antibodies targeting Pvs230D1M (**Figure S3**). For this experiment sera from 26 USA donors were used as negative controls.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad8). Analyses were performed using data from two independent experiments. We considered serum reactivity levels above a maximum threshold of 3 standard deviations from the geometric mean for the study population to be unreliable, and hence excluded one Brazilian subject from the analyses. Correlation analyses were tested using logistic-regression analysis. One-Way ANOVA followed by multiple comparisons test was employed to compare different groups, when applicable. For **Figures 4**, **5**, Kruskal-Wallis test was performed. Significance level used was $p < 0.05$ for all statistical analyses.

Map Design

Maps were created using *maptools* and *raster* packages and plotted using *ggplot2* package of the R software (http://www.r-project.org, version 3.5.3).

RESULTS

Anti-Pvs230D1M Antibody Response Is Induced During Malaria Infection

To assess humoral response to sexual stage antigens in subjects living in areas of malaria transmission, sera/plasma samples obtained from patients presenting with acute *P. vivax* infection diagnosed by blood smear microscopy were examined for IgG levels against Pvs230D1M. Sera from 36 healthy non-immune USA donors were used as negative controls to determine the cut-off OD value (1.18). The seroprevalence of IgG antibodies with specificity for Pvs230D1M was 27.1% in Brazil (19/70 samples) and 26.6% in Cambodia (21/79 samples) **(Figure 2).**

IgG1 and IgG3 Are the Most Prevalent IgG Subtype Responses to Pvs230D1M

To evaluate differential representation in immune response to Pvs230 among the four human IgG subtypes, we evaluated IgG1, 2, 3, and 4 responses against Pvs230D1M. Detectable IgG3 levels (19.3% in Brazil and 20.6% in Cambodia) and IgG1 levels (10.5% in Brazil and 15.1% in Cambodia) were most frequent, with limited IgG2 responses (5.3% in Brazil and 1.4% in Cambodia). The frequency of IgG4 responses was 0% at both study sites **(Figure 3)**.

Pvs230D1M IgG Response Increases With Age in Cambodian Subjects

Although no direct correlation was observed between age and Pvs230D1M IgG titers **(Figure 4)**, a cumulative effect of age in Pvs230D1M antibody response was observed in Cambodia. Seroprevalence for Pvs230D1M was higher with increasing age strata in Cambodian subjects: 1.3% Pvs230D1M IgG responders among 1–9 year-olds; 6.3% among 10–19 year-olds; and 19.0%

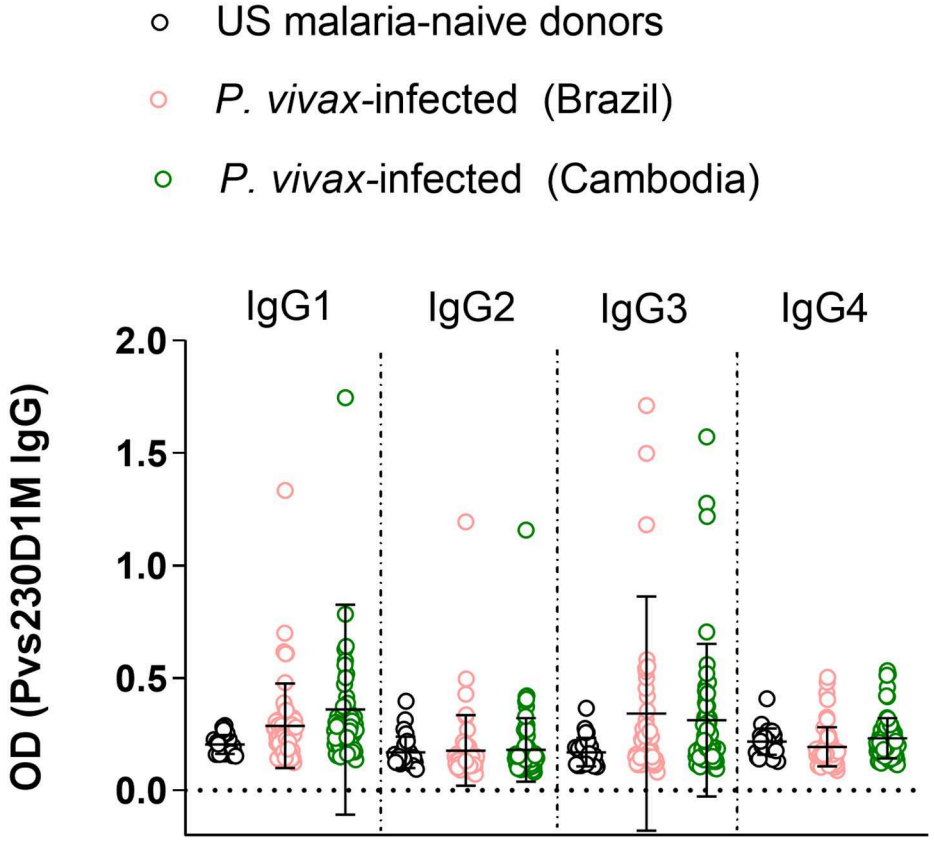

FIGURE 3 | Immunoglobulin G subclass responses to Pvs230D1M in Brazil and Cambodia. Healthy malaria-naïve donor sera were used to define the background for each subclass, and cut-off was calculated based on mean control + 3 standard deviations. IgG3 and IgG1 responses were predominant with limited IgG2 and no IgG4 response. One-Way ANOVA followed by multiple comparisons was used for this analysis and results are displayed as mean ± SD.

among 20 years old and above (1–9 years group vs. 20 years and older, $p = 0.021$, **Figure 4**). Pvs230D1M titers were not evaluated for correlation with age in Brazil since the median age was 45 years old and no samples were obtained from children.

Anti-Pvs230D1M IgG Titers Do Not Correlate With Parasitemia Levels

We assessed the correlation of anti-Pvs230D1M specific antibodies with parasitemia levels. There was no significant association between Pvs230D1M IgG response and asexual parasitemia in subjects from Brazil ($p = 0.38$) or Cambodia ($p = 0.43$) **(Figure 5)**.

Increased Pvs230D1M IgG Titers Correlate With Decreased Hemoglobin in Brazil, but Not in Cambodia

Despite its historical designation as "benign tertian malaria," *P. vivax* has received increased attention as a cause of severe sequelae, including severe anemia (24, 25). We assessed whether *P. vivax* antibody levels are inversely correlated with hemoglobin levels, to support the hypothesis that anemia in either of these populations may be due to *P. vivax* infections. Antibodies elicited in response to Pvs230D1M were negatively correlated with hemoglobin levels in Brazilian subjects ($r = -0.3906$, $p = 0.0168$), but not in Cambodian subjects **(Figure 6)**.

Increased PvDBP-RII and PvCSP IgG Titers Correlate With Decreased Hemoglobin in Brazil

To assess whether the correlation of hemoglobin and antibody titers is specific to Pvs230D1M IgG, we analyzed the relationships with *P. vivax* proteins PvCSP (sporozoite stage protein) and PvDBP-RII (merozoite stage protein). The seroprevalence of antibodies with specificity for PvDBP-RII was 67.1% (47/70 samples) in Brazil and 48.1% (38/79 samples) in Cambodia, and for PvCSP 11.4% (8/70 samples) and 41.8% (33/79 samples) respectively **(Figure 7)**. Hemoglobin levels negatively correlated with PvDBP-RII ($r = -0.4100$, $p = 0.0086$) and PvCSP ($r = -0.3554$, $p = 0.0247$) IgG titers in Brazil **(Figure 8)**, but no correlations were seen in Cambodia, indicating that the relationships to hemoglobin are similar for seroreactivities against liver stage, blood stage, and sexual stage antigens within the two study populations. Antibody levels against liver and

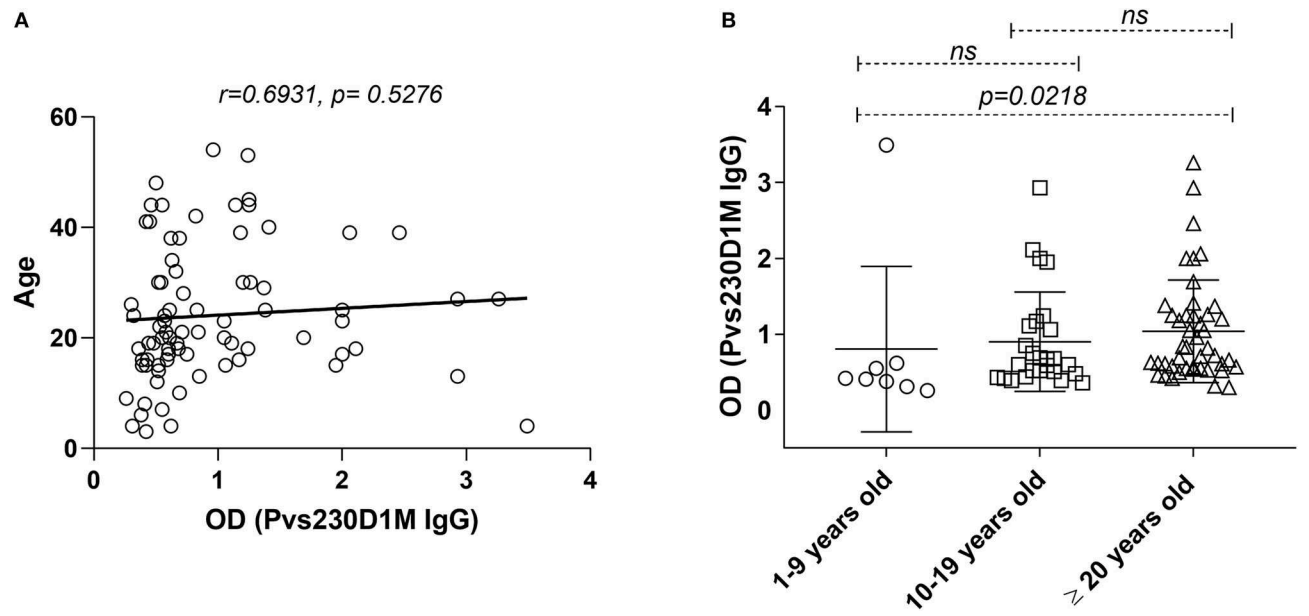

FIGURE 4 | Pvs230D1M IgG responses do not correlate with age in Cambodia but increase significantly within age strata. **(A)** Pearson correlation and Linear regression, comparing Pvs230D1M antibody titers and age. **(B)** Seroprevalence for Pvs230D1M IgG (mean ± SD) was increased with age strata in Cambodian subjects: Pvs230D1M IgG response detected in 1.3% of 1–9 year old children ($N = 8$), 6.3% in 10–19 year old children ($N = 26$), and 19% in adults 20 years and older ($N = 45$). Kruskal-Wallis statistic test was used for the comparisons.

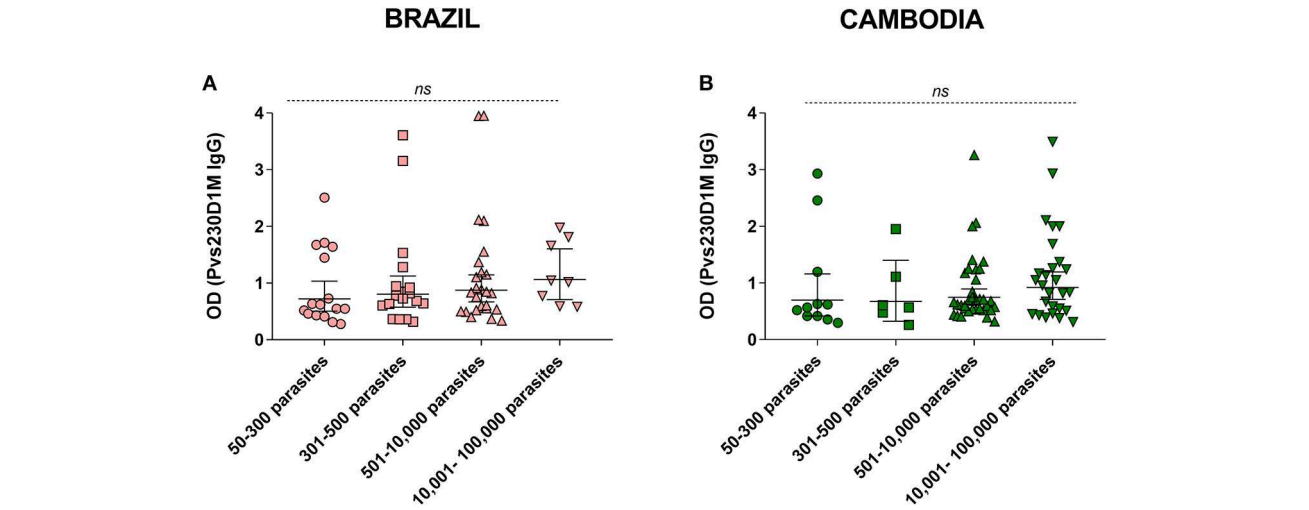

FIGURE 5 | Parasitemia levels in comparison to Pvs230D1M IgG titers in **(A)** Brazil and **(B)** Cambodia. Kruskal-Wallis analysis revealed no significant difference between the groups. Detailed information on parasitemia levels for each subject is reported in **Table S1**.

blood stage proteins did not significantly correlate with age in Brazil or Cambodia (**Figure S1**).

Antibodies to Pfs230D1M, *P. falciparum* Ortholog of Pvs230D1M, in *P. vivax*-Infected Subjects

We investigated whether antibody responses during *P. vivax* infection might also be reactive against Pfs230D1M. We found that 7.2% of the sera from *P. vivax*-infected subjects from Brazil and 16.5% from Cambodia had detectable antibody against Pfs230D1M **(Figure 7)**.

Concurrent Antibody Responses to Pvs230D1M and Pfs230D1M Are Not Due to Shared Epitopes

Due to similarities between Pvs230D1M and Pfs230D1M protein sequences, and the fact that *P. falciparum* malaria cases are also present in Brazil and Cambodia, we examined whether subjects may have developed antibody responses to both

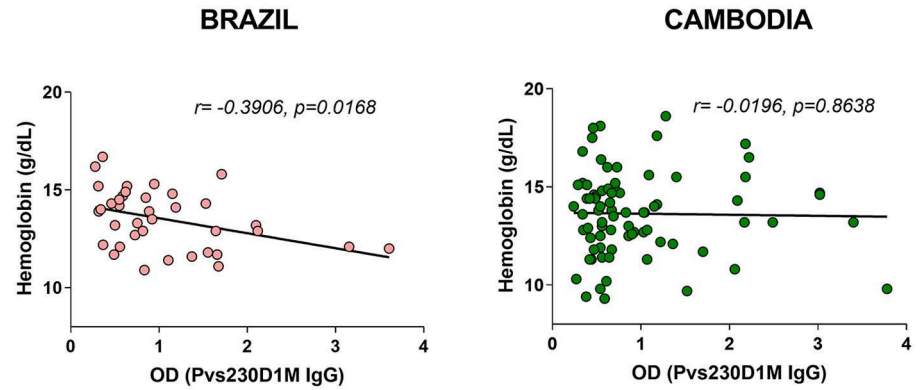

FIGURE 6 | Pvs230D1M-specific antibody responses inversely correlate with hemoglobin levels in Brazil but not in Cambodia.

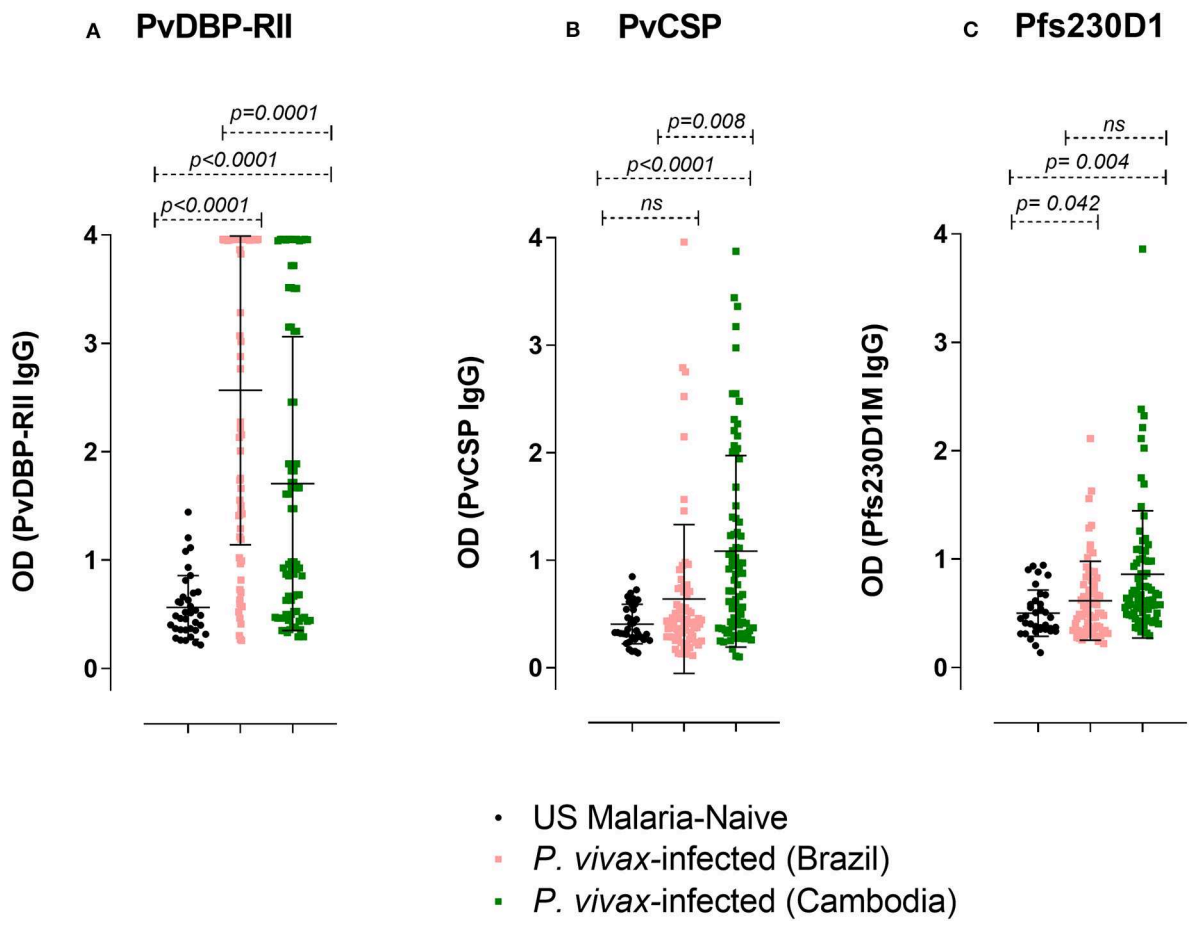

FIGURE 7 | IgG ELISA levels against PvDBP-RII, PvCSP, and Pfs230D1M in *P. vivax*-infected subjects. The seroprevalence of IgG antibodies with specificity for **(A)** PvDBP-RII in Brazil was 67.1% (47/70 subjects) and in Cambodia 48.1% (38/79 subjects); **(B)** PvCSP in Brazil was 11.4% (8/70 subjects) and in Cambodia 41.8% (33/79 subjects); **(C)** Pfs230D1M in Brazil was 7.2% (5/69 subjects; the volume of one sample was insufficient for assay) and in Cambodia 16.5% (13/79). The cut-off levels for detection (1.44, 0.97, and 1.16 respectively for PvDBP-RII, PvCSP, and Pfs230D1M ELISA) were based on control mean + 3SD calculation. One-Way ANOVA followed by multiple comparisons was used for this analysis and results are displayed as mean ± SD.

Pvs230D1 and Pfs230D1. Three (4.3%) subjects in Brazil and six (7.6%) subjects in Cambodia presented with concurrent antibody responses to Pvs230D1 and Pfs230D1. We assayed Pvs230D1M-depleted sera to investigate whether Pfs230D1M titers resulted from cross-reactive epitopes with Pvs230D1M. After depletion assay to remove antibodies specifically generated

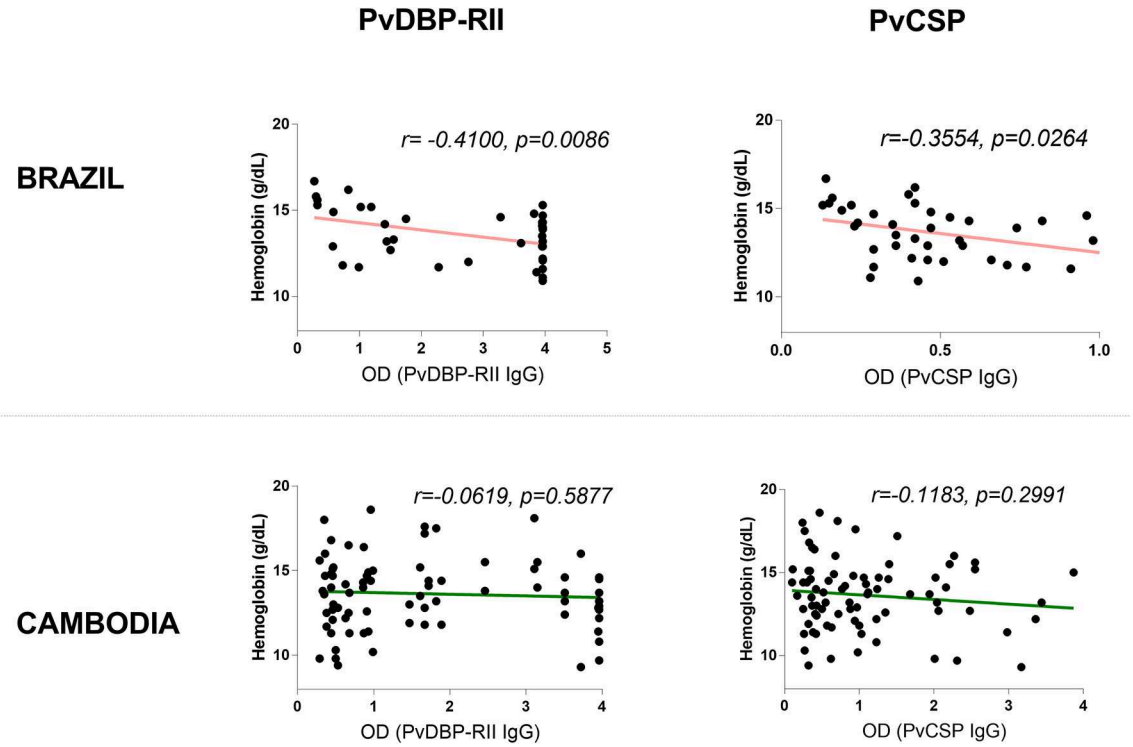

FIGURE 8 | Correlation between antibodies elicited in response to blood-stage antigen (PvDBP-RII) or sporozoite-stage antigen (PvCSP) and hemoglobin levels in Brazil and Cambodia.

against Pvs230 (**Figure S2**), Brazilian and Cambodian samples maintained IgG levels to Pfs230D1M comparable to pre-depletion levels **(Figure 9)**, suggesting that responses were not due to cross-reactive epitopes. Pfs230D1M antibody titers may therefore be due to microscopically undetected co-infection with *P. falciparum* or previous exposure to *P. falciparum* in these populations.

DISCUSSION

Antibodies to sexual stage parasites can be induced in response to infection (9, 10, 26). Compared to *P. vivax,* the antibody response to *P. falciparum* sexual stages is better characterized and it is known that children and adults in endemic areas acquire an immune response to Pfs230 (8). However, the naturally acquired response to Pvs230 has not been characterized, despite *P. vivax* being responsible for the majority of malaria cases in Latin America and in Southeast Asia (1). Understanding adaptive immune responses to *P. vivax* antigens present in sexual-stage parasites in the mosquito and human host can contribute to development of transmission-blocking strategies. In the current study, the prevalence of antibodies against domain 1 of Pvs230 (Pvs230D1M) during *P. vivax* infection was 27.1% in Brazil and 26.6% in Cambodia. Similarly, previous studies have shown that the ortholog Pfs230 reacted to sera from 28.6% of malaria-exposed adults in an area of seasonal transmission in Burkina Faso and from 20.7% of *P. falciparum*-infected donors in a low endemic area of Tanzania (8, 10).

Although anti-Pfs230 antibody activity can be enhanced by complement (27, 28), information on IgG subclasses generated against Pvs230 in naturally infected humans has not been described. Previous work showed that sera from mice immunized with Pvs230 reduces the number of oocysts in midguts of mosquitoes fed with blood from *P. vivax*-infected subjects, and this reduction occurs in the presence or absence of complement (18). We evaluated whether the natural antibody response to Pvs230D1M in humans would be characterized by higher levels of complement-fixing IgG subclasses. In our analyses of Pv230D1M IgG subclass frequency, IgG1 and IgG3 were shown to be the predominant subclasses during malaria infection and these isotypes are known to fix complement (29–33). This suggests that the functional activity of naturally acquired anti-Pvs230 antibody might be enhanced by complement, but this requires further investigation.

Although the correlation between age and Pvs230D1M IgG was not statistically significant, Pvs230D1M-specific antibody titers in Cambodia differed ($p = 0.021$) between 1–9 year old subjects vs. subjects ≥20 years old. These results need to be interpreted in the context of characteristics of the study sites. In Pursat province, Cambodia, exposure to malaria frequently occurs as a result of occupation and exposure is low in children. A longitudinal study must be conducted with a larger number of samples collected from high and low endemic areas to confirm the age-cumulative effect of Pvs230 IgG response and its relationship with high or low transmission areas. Previous studies have suggested that IgG responses against sexual stage *P. falciparum* proteins do not increase with age (8–10, 26). For

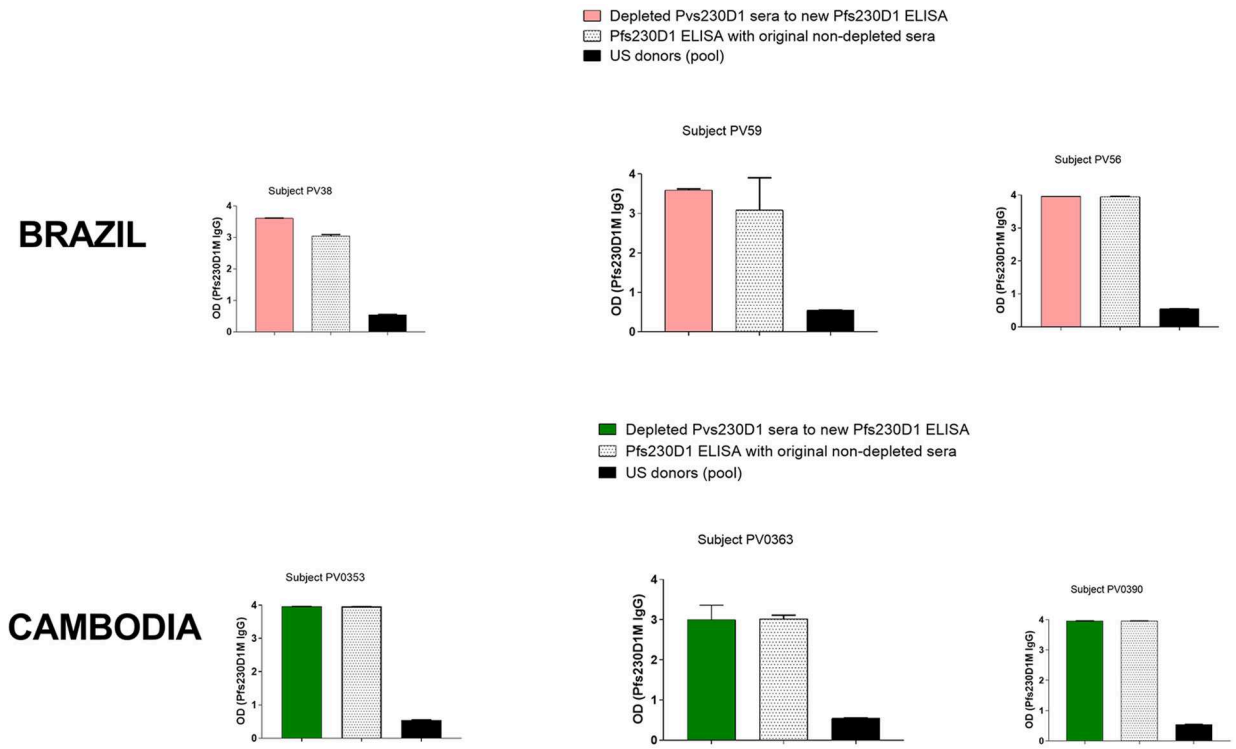

FIGURE 9 | ELISA on Pvs230D1M-depleted sera. Pvs230D1M IgG levels were gradually reduced in sera by depletion assay (**Figure S2**), and then samples were submitted to ELISA against Pfs230D1M. Specificity of depletion was confirmed measuring antibody titers to PvDBP-RII in sera depleted for Pvs230 IgG (**Figure S3**).

example, a study performed in a low *P. falciparum* transmission area in Tanzania did not reveal correlations between antibodies generated in response to Pfs230 or to Pfs48/45 and age (10).

P. vivax is associated with lower hemoglobin concentration and can cause severe malaria (34–37). Here, we found an inverse correlation between anti-Pvs230D1M antibody titers and hemoglobin levels in Brazil. Confirming that low levels of hemoglobin were due to malaria, we observed the same correlation with PvDBP-RII and PvCSP IgGs (elicited in response to blood stage and pre-erythrocytic stage parasites, respectively). PvDBP-RII titers were higher in Brazil than in Cambodia, supporting our hypothesis that exposure in Rondônia state may be higher than in Pursat province, Cambodia. Intriguingly, PvCSP titers were higher in Cambodia than in Brazil, which may be attributable to the fact that the PvCSP recombinant protein was based on a parasite strain isolated in Iran (VK210), a country in Asia, closer to Cambodia than to Brazil.

In Cambodia, no correlation was observed between antibody titers and hemoglobin levels. We hypothesize that high endemicity with more frequent infections in a region such as Brazil lowers hemoglobin levels and therefore negatively correlates with increased antibody levels, while low endemicity in a region such as Cambodia entails more sporadic infections with potentially less impact on hemoglobin levels. Of note, the ranges of hemoglobin levels were similar at the two study sites, as were the proportions of male and female subjects. Hemoglobin levels assessed prior to infection were not determined, since those samples were not available for this study.

We found no relationship of anti-Pvs230D1M antibody to level of parasitemia in Brazil ($r = 0.060$, $p = 0.6469$) or Cambodia ($r = 0.1193$, $p = 0.2948$). Data on gametocytemia were not collected at the time of blood smear microscopy and therefore are not available for analysis. In future, it will be of interest to perform a longitudinal study, to evaluate serologic parameters identified before, during and after infection and to correlate *P. vivax* sexual stage antibody responses to gametocyte carriage.

P. vivax-infected subjects from Brazil and Cambodia displayed antibodies against Pfs230D1M, the ortholog of Pvs230D1M in *P. falciparum*. ELISA on Pvs230D1M-depleted sera suggests that Pfs230D1M titers were produced in response to *P. falciparum* pre-exposure or co-infection. The Pfs230D1M IgG response was more frequent in Cambodia than Brazil, perhaps reflecting the greater proportion of malaria infections caused by *P. falciparum* in Cambodia vs. Brazil (58 vs. <10%) (1). Since *P. falciparum* infection is known to cause anemia (38, 39), a mixed infection could influence the correlation of hemoglobin with antibody titers. A limitation in our study was that the low volume of plasma samples precluded determination of functional activity of Pfs230-purified IgG in Standard Membrane Feeding Assay (SMFA) that assesses the reduction of *P. falciparum* parasite transmission to mosquitoes.

Our findings provide a first characterization of naturally acquired antibody responses to Pvs230 among *P. vivax*-infected subjects from regions of differing transmission intensity in Brazil and Cambodia.

DATA AVAILABILITY STATEMENT

Datasets generated for this study are included in the manuscript/**Supplementary Files**. Additional datasets are also available upon request.

ETHICS STATEMENT

The studies involving human participants in Brazil were reviewed and approved by the Centro de Pesquisa em Medicina Tropical (CAAEs: 0008.0.046.000-11, 0449.0.203.000-09) and the Ethics Committee of the Federal University of Minas Gerais (CAAE: 27466214.0.0000.5149), Brazil. The human study in Cambodia was approved by the Institutional Review Board (IRB), NIAID, NIH, and National Ethics Committee for Human Research (NECHR), Cambodia (ClinicalTrials.gov Identifier: NCT00663546). Written informed consent was obtained from each participant. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CC and PD conceptualized and supervised the study. CA, SSu, SSr, DP, LB, and RF coordinated the clinical study. CA, LB, and RF obtained the samples. BT performed the experiments. NM, NS, DN, and NT provided recombinant proteins. CC, BT, NA, and JK performed the analyses. All authors interpreted the data. BT, CC, and PD wrote the manuscript, with input from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02295/full#supplementary-material

Table S1 | Background data for all subjects from Brazil and Cambodia. Information on sex, age, hemoglobin levels, and parasitemia. Parasitemia data in Brazil, originally reported in semi-quantitative format, were converted to parasites per mm3 to be compared with data from Cambodia, reported as parasites/mm3. The conversions were: ½+ = 50–300 parasites/mm3; + = 301–500 parasites per mm3; ++ = 501–10,000 parasites/mm3; +++ = 10,001–100,000 parasites.

Figure S1 | Correlation between age and antibody titers against PvDBP-RII, PvCSP, and Pfs230D1M in Cambodia.

Figure S2 | Confirmation of depletion (or reduction) of Pvs230D1M IgG from sera used to evaluate Pfs230D1M IgG levels.

Figure S3 | Specificity of Pvs230D1M IgG depletion. PvDBP-RII IgG levels remain unaltered after depletion of Pvs230D1M IgG.

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Exploring *Plasmodium falciparum Var* Gene Expression to Assess Host Selection Pressure on Parasites During Infancy

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In sub-Saharan Africa, children below 5 years bear the greatest burden of severe malaria because they lack naturally acquired immunity that develops following repeated exposure to infections by *Plasmodium falciparum*. Antibodies to the surface of *P. falciparum* infected erythrocytes (IE) play an important role in this immunity. In children under the age of 6 months, relative protection from severe malaria is observed and this is thought to be partly due to trans-placental acquired protective maternal antibodies. However, the protective effect of maternal antibodies has not been fully established, especially the role of antibodies to variant surface antigens (VSA) expressed on IE. Here, we assessed the immune pressure on parasites infecting infants using markers associated with the acquisition of naturally acquired immunity to surface antigens. We hypothesized that, if maternal antibodies to VSA imposed a selection pressure on parasites, then the expression of a relatively conserved subset of *var* genes called group A *var* genes in infants should change with waning maternal antibodies. To test this, we compared their expression in parasites from children between 0 and 12 months and above 12 months of age. The transcript quantity and the proportional expression of group A *var* subgroup, including those containing domain cassette 13, were positively associated with age during the first year of life, which contrasts with above 12 months. This was accompanied by a decline in infected erythrocyte surface antibodies and an increase in parasitemia during this period. The observed increase in group A *var* gene expression with age in the first year of life, when the maternal antibodies are waning and before acquisition of naturally acquired antibodies with repeated exposure, is consistent with the idea that maternally acquired antibodies impose a selection pressure on parasites that infect infants and may play a role in protecting these infants against severe malaria.

Keywords: malaria, maternal antibodies, var gene expression, age, infants

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INTRODUCTION

In many malaria endemic regions, incidence of severe malaria is rare in children younger than 6 months (1, 2) suggesting a potential role of maternal antibodies in protection from malaria. In a cross sectional survey in Benin and Gambia, it was reported that the mean parasite densities among infants younger than 1 year was significantly lower than in children aged between 1 and 9 years (3). Several studies have explored the role of antibodies in this phenomenon (4–8). However, because of the difficulty in conclusively linking antibody carriage to clinical protection, the mechanism of protection and the targets of the maternal antibodies remain unclear.

An alternative approach to explore the potential role of host antibodies, is to explore the parasites that cause infections in infants and seek parasite markers that will act as a read-out of *in vivo* antibody selection on the infecting parasites populations. The *Pf*EMP1 family of antigens that are expressed on the surface of parasite infected erythrocytes provide a potentially useful marker for this purpose. *Pf*EMP1 is a highly diverse family of multi-domain parasite antigens that are inserted into the infected erythrocyte surface, encoded by a family of about 60 *var* genes in every genome (9, 10). *Pf*EMP1 mediates the binding of Infected Erythrocytes (IE) to vascular endothelial cells, allowing the parasite to sequester in the microvasculature of the organs and avoid clearance by the spleen (11). Therefore, through sequestration, *Pf*EMP1 supports parasite growth and plays an important role in the pathogenesis of malaria.

Despite their overall high level of diversity, parasites encode subsets of *Pf*EMP1 antigens that are relatively conserved. A subset of *Pf*EMP1 referred to as group A and those containing commonly occurring domain arrangements called domain cassettes 8 and 13 (DC8 and DC13) are thought to play a role in the pathogenesis of malaria (12, 13). *In vitro* studies have shown that these subsets of *Pf*EMP1 mediate strong binding to endothelial cells (14) and to non-infected erythrocytes in a process referred to as rosetting (15, 16). Therefore, through their cytoadhesive properties, they have been hypothesized to support parasite growth, increasing the risk of severe malaria. Further, these *var* subsets are more commonly expressed in parasites from children with low host immunity and those with severe malaria (17, 18). Although most studies on clinical *P. falciparum* isolates have found that host age is negatively correlated with expression of group A and DC8 *var* genes (18–20), these studies have not considered *var* expression in parasites sampled from children with malaria below 12 months, possibly due to the extreme rarity of *P. falciparum* infection in children in this age group (21). In the study described here, we aimed to overcome the rarity of parasites sampled from infants by making use of a large collection of parasite isolates that have been collected over a 16-year period. We hypothesized that if maternal antibodies are important in the protection of children from malaria in early life, there will be a positive association between the expression of group A *var* genes in parasites and the age of the children in the first 12 months of life, as maternal antibodies wane.

MATERIALS AND METHODS

Study Site, Sample Collection, and Ethics

The study was carried out at Kilifi County which is situated on the Kenyan coast. Parasite isolates and plasma samples collected between 1994 and 2012, from *Plasmodium falciparum* positive pediatric admissions and longitudinal cohort children, were used for the study. Ethical approval was obtained from the Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI/SERU) under the protocol; KEMRI/SERU/3149, and informed consent was obtained from the parents/guardians of the children.

Var Expression Analysis

RNA was obtained from TRIzol™ reagent (Invitrogen, catalog number 15596026) preserved *P. falciparum* positive venous blood samples, obtained from the children recruited for the study. RNA was extracted using a Chloroform method (19) and cDNA was synthesized using the Superscript III kit (Invitrogen, catalog number 18091050) following the manufacturer's protocol. *Var* gene expression analysis was carried out through (a) PCR amplification of a conserved region of the *var* genes (expressed sequence tags) and sequencing using capillary and 454 platforms, and (b) quantitative real-time PCR as described below.

a) Expressed sequence Tag (EST) sequencing

Using degenerate AF and BR primers (22), EST consisting of the conserved DBLα region of the *var* genes were amplified from the cDNA of each isolate by PCR. The PCR product was cleaned and sequenced as described below.

i. ***454 sequencing and sequence assembly***

To enable sequencing, a multiple identifier (MID) tag was ligated onto PCR products from each isolate. A total of 48 MID tags were available allowing for pooling of 48 different isolates before sequencing using the 454 GS-FLX 3 Titanium technology using the standard protocol (Roche). For each pool, sequencing success was determined using the amount of data and length distribution of reads at the end of the sequencing run. Pools that did not meet these criteria were resequenced.

Reads from the data were partitioned using their MID and pool identity. Reads shorter than 300 bp were excluded from further analysis. The following methods were used independently for each set of reads. The reads were assembled with cap3 (version date 12/21/07) using the default settings (23). The resulting contigs were clustered by putting contigs with BLAST (version 2.2.25) matches of at least 98% identity and 95% match length of the longest sequence into the same cluster. Each cluster of contigs was assembled using cap3 with the default settings (23). A ruby program bio-dbl-classifier was used for classification of each of the assembled contigs into their respective DBLα subgroup as described in Bull et al. (24). Concurrently, the level of expression of each assembled contig was determined by mapping all the reads from that isolate to the assembled consensus contig sequences from the same isolate at a 95% identity. The number of reads per contig, DBLα classification file and the total number of reads for each isolate were then used to determine the proportion of

expression of each of the different DBLα subgroups in each isolate using STATA as follows e.g., for each isolate,

$$\%\ expression\ of\ Group\ A-like = \frac{no\ of\ contigs\ classified\ as\ group\ A\ -\ like}{total\ no\ of\ reads} \times 100 \quad (1)$$

ii. ***EST sequencing by capillary***

For 237 samples, the capillary sequencing platform was used to sequence the amplified EST as described (18, 24). The *var* expression data published in these studies are included in this study.

b) Var transcript quantification using RT-PCR

Real-time PCR data was obtained as described (17, 25, 26). Four primers targeting DC8 (named dc8-1, dc8-2, dc8-3, dc8-4), one primer targeting DC13 (dc13) and two primers targeting the majority of group A *var* genes (gpA1 and gpA2) were used in real-time PCR analysis (**Table S1**). We also used two primers, b1 and c2, targeting group B and C *var* genes, respectively (27) (**Table S1**). Two housekeeping genes, Seryl tRNA synthetase and Fructose bisphosphate aldolase (20, 28, 29) were used for relative quantification of the expressed *var* genes. The PCR reaction and cycling conditions were carried out as described in Lavstsen et al. (20) with the Applied Biosystems 7500 Real-time PCR system. We set the cycle threshold (Ct) at 0.025. Controls with no template were included at the end of each batch of 22 samples per primer and the melt-curves analyzed for non-specific amplification. Genomic DNA from the IT4 laboratory parasite line at 10 ng/μl was used as a standard sample in all plates. The var gene "transcript quantity" was determined relative to the mean transcript of the two housekeeping genes, Seryl tRNA synthetase and Fructose biphosphate aldolase as described (20). For each test primer, the Δct for both the test samples and the standard genomic DNA was calculated and used to generate the ΔΔct value which was then transformed to arbitrary transcript unit (Tu_s) using the formula [$Tu_s = 2^{(5-\Delta\Delta ct)}$]. However, we also estimated "proportional expression" of the transcripts within each sample. When calculating proportional expression from qPCR (described under "statistical analysis" below), Tu_s calculated as described in Lavstsen et al. (20) i.e., $2^{(5-\Delta ct)}$ were used. We assigned a zero Tu_s value if a reaction did not result in detectable amplification after 40 cycles of amplification, i.e., if the Ct value was undetermined.

Flow Cytometry

We had a measure of antibodies to infected erythrocytes (αIE) for 215 children from a previously published work (18), of which 27 were aged between 0 and 12 months and the rest were older than 12 months. To boost the αIE data for the children aged between 0 and 12 months, we measured αIE levels for 69 children who had available archived acute plasma samples. The antibody level that each child had at the time of parasite sampling was determined using Flow cytometry against one *P. falciparum* clinical isolate matured *ex vivo* to the trophozoite stage and cryopreserved in liquid nitrogen as described in Warimwe et al. (18). The cryopreserved trophozoites were thawed as previously described (30), erythrocyte pellet volume estimated, parasitemia determined by microscopy and diluted to 1% using fresh uninfected erythrocytes. The cells were resuspended in 0.5% bovine serum albumin in 1% phosphate buffered saline (0.5% BSA/PBS) such that 11.5 μl of the mixture contained 0.5 μl of the cells. The reaction mixture was stained with Ethidium bromide at a final concentration of 10 μg/ml and 11.5 μl of the mixture per well was aliquoted into U-bottom 96-well plates (Falcon®, catalog number BD 353077). One microliter of each child's acute plasma was pipetted into separate wells and the mixture incubated for 30 min at room temperature. Following three washes with 0.5% BSA/PBS, the cells were incubated in the dark for 30 min at room temperature with 50 μl/well of fluorescein isothiocynate (FITC)-conjugated sheep anti-human IgG (Binding Site, catalog number AF004.X) diluted 1:50 in 0.5% BSA/PBS. Following three washes, the cells were diluted 10-fold in 0.5% BSA/PBS and at least 1000 IE acquired on a BD FACs Canto II cytometer (BD Biosciences). Plasma from four non-exposed European donors and one from a semi-immune Kilifi adult were included in each plate and used as negative and positive controls, respectively.

Data analysis was done using FlowJo v10 software (TreeStar Inc.). Following initial gating to remove singlets, IE were distinguished from uninfected erythrocytes on the basis of their Ethidum Bromide staining. Next, median fluorescence intensity on the FITC channel from each isolate was obtained by subtracting the signal of the uninfected from the infected erythrocytes signal (**Figure S1**). To cater for non-specific binding, the mean median fluorescent intensity of the four non-exposed sera on each plate was subtracted from the median florescence intensities of each child's sample.

Statistical Analysis

The median transcript units from quantitative real time PCR were calculated as follows; DC8 median from four primers used (dc8-1, dc8-2, dc8-3 and dc8-4) and group A median from two primers (gpA1 and gpA2). The proportion of transcripts for each *var* group was then calculated from the sum of transcript (sum of DC8 median, Group A median, DC13, B1, and C2) e.g.,

$$DC8\ proportion = \frac{DC8\ median}{sum\ of\ transcript} \quad (2)$$

Spearman's rank correlation was used to determine the strength of the correlations between different variables (e.g., *var* gene expression with host age and host αIE). Scatter plots were used to visualize the direction of correlations between *var* expression and various variables including host age and αIE levels. All analysis was done using Stata V13.

RESULTS

We used an expressed sequence tag approach (EST) to broadly look at the proportional expression of different subsets of *var*

genes. In addition, we used a real time PCR approach using primers designed to detect various subsets of *var* genes. The number of isolates whose *var* expression was analyzed using the two approaches and more information about the primers used is summarized in **Table S1**.

Parasite Group A *Var* Gene Expression Increases With Age in the First Year of Life

We and others have previously reported negative correlations between age and the expression of group A-like *var* genes measured using the EST approach (15, 18, 19, 31). A similar observation was made when expression of group A and DC8 was quantified by qPCR (17). In this study, we looked at the correlation between age and *var* gene expression in the first year of life and thereafter. We found that the expression of cys2 and group A-like *var* genes, as determined from EST, was positively correlated with host age in the first 12 months (cys2; $p = 0.005$, group A-like; $p = 0.0004$, **Figure 1A**, **Figures S2A,B**). Similarly, positive correlations were found with the transcript quantity of group A *var* genes determined with both gpA1 and gpA2 primers (**Table S1**) designed to target broad group A *var* genes (gpA1; $p = 0.00007$, gpA2; $p = 0.003$, **Figure 1B**, **Figures S2C,D**). The relationships between the expression of group A *var* genes and host age remained significant after Bonferroni correction for 10 multiple comparisons ($p = 0.0007$, and $p = 0.03$ for gpA1, and gpA2, respectively). In the first year of life, expression of DC13 was positively associated with host age ($p < 0.005$, **Figure 1B**, **Figure S2E**) though not significant after correction for 10 multiple comparison ($p = 0.05$). The expression of group B *var* genes as determined with b1 (**Figure 1B**, **Figure S2J**) and DC8 primers did not show significant association with host age in the first year of life (**Figure 1B**, **Figures S2F,G**). Unexpectedly, expression of group C *var* genes (c2) showed positive associations with host age ($p < 0.02$, **Figure 1B**, **Figure S2K**).

We hypothesized that the positive correlation between the expression of group A *var* genes and host age in the first year of life was due to the decay of maternal antibodies. If this were the case, an opposite effect would be expected in children above 12 months as they become exposed to malaria and make antibodies. In support of this, the transcript quantity of group A *var* genes (except DC13) in children over 12 months was negatively associated with host age (group A-like; $p = 0.004$, gpA1; $p = 0.0016$, gpA2; $p = 0.0004$, **Figures 1A,B**, **Figures S2A–D**). The relationship between host age and the transcript quantity of DC8 *var* genes, as expected, was in the negative direction (**Figure 1B**, **Figures S2F–I**).

In an alternative approach, the proportion of group A, B, C, and DC8 *var* gene transcript was calculated from the estimated overall transcript quantity of group A, DC13, DC8, group B, and C (17). In this analysis, only a subset of 654 observations that had expression data for all the *var* subsets measured and age were included (**Table S1**). The proportion of group B *var* genes (b1_prop) was negatively associated with host age in the first year of life ($p = 0.01$, **Figure 1B**), and positively associated with host age in children older than 12 months ($p = 0.002$, **Figure 1B**). In contrast, the proportional expression of group A *var* genes (gpA_prop) was positively associated with host age in the first year of life ($p = 0.004$, **Figure 1B**) and negatively associated with host age in children older than 12 months ($p = 0.00004$, **Figure 1B**). DC8 proportional expression association with host age in the first year of life was not significant (**Figure 1B**) but showed weak significant association after 12 months of life (**Figure 1B**).

Group C proportional expression was not associated with age in the first 12 months but was positively associated with age after 12 months ($p = 0.0001$, **Figure 1B**).

Antibodies to Infected Erythrocyte Surface Antigens (αIE) Showed Negative and Positive Associations With Host Age in Children Up to 6 Months and Above 12 Months, Respectively

In malaria endemic regions, it is expected that there is a positive correlation between host age and antibody levels (18). For this study, due to the relatively rapid decay in maternal antibodies during the first 6 months of life and slow simultaneous acquisition of antibodies following exposure to malaria, we hypothesized that overall there will be a decline in host antibodies with age in the first year, particularly in the first 6 months. Consistent with waning maternal antibodies, the αIE levels was negatively correlated with host age, especially in the first 6 months ($p = 0.008$, **Figure 1C**). In contrast, positive correlation between host age and αIE in children older than 12 months was observed ($p < 0.00001$, **Figure 1C**).

αIE Were Negatively Associated With Expression of Group A and DC8 *Var* Gene Subsets

As expected, we found significant negative correlations between group A expression (both transcript quantity and proportional expression) and αIE in both 0–12 months and above 12 months old children (**Figures 1A,B**). Similar correlation was also observed between DC8 expression and αIE (**Figure 1B**). The correlation between group B (b1) transcript quantity and αIE was in the negative direction but significant only in the children older than 12 months (**Figure 1B**) while group C (c2) transcript quantity showed no association with αIE (**Figure 1B**). The proportional expression of group B (b1_prop) and C (c2_prop) were also not significantly correlated with αIE in the children 0–12 months old, however, group C proportional expression showed significant positive correlation with αIE (**Figure 1B**). This is in contrast to group A(gpA_prop) and DC8 (dc8_prop) proportional expression which were in the negative direction (**Figure 1B**). The correlation between *var* gene expression and αIE in children aged 0–6 months was consistent with that of 0–12 months, although the association was not reaching statistical significance, most likely due to small sample size (**Figures 1A,B**).

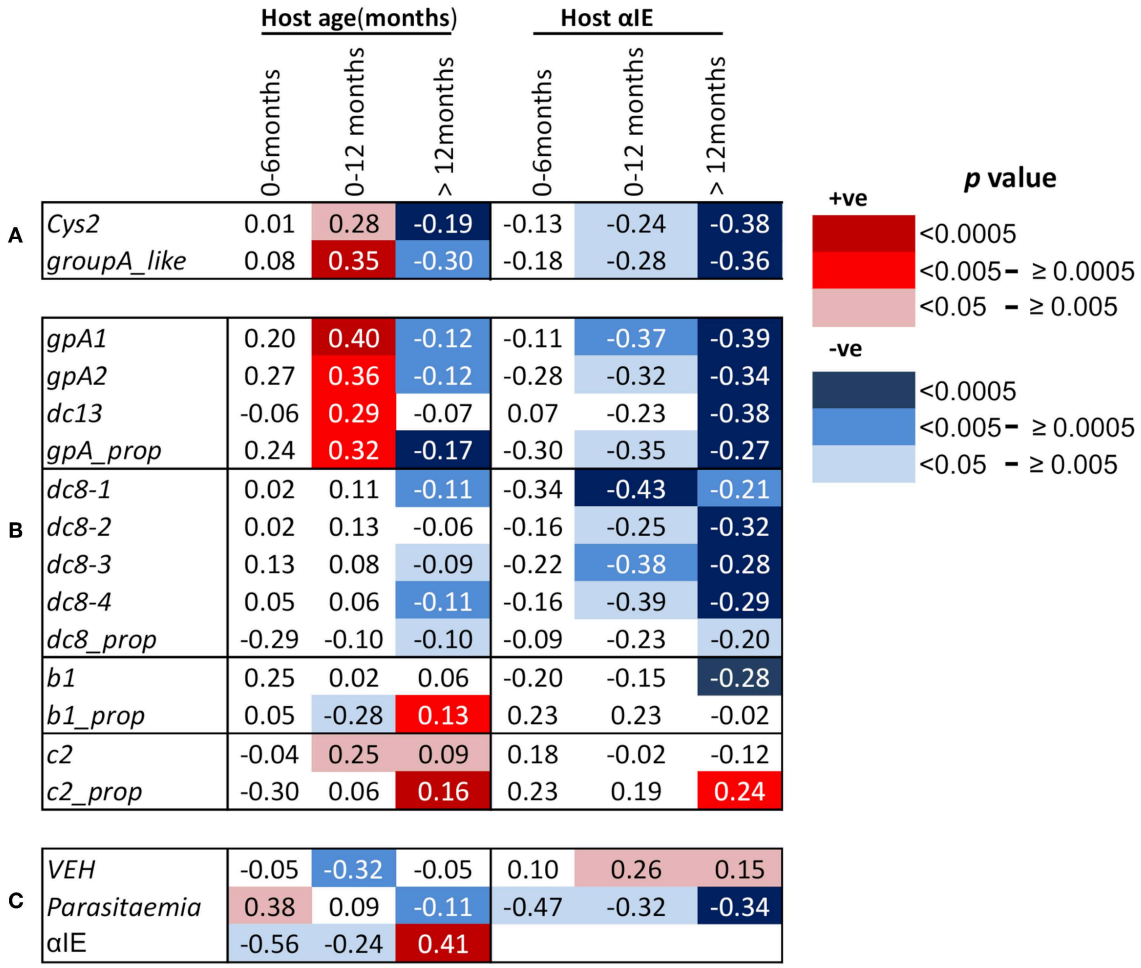

FIGURE 1 | Correlations between host age in months, host antibodies to infected erythrocytes (αIE) and *var* gene expression of the infecting parasites. Shown are Spearman's rank correlation coefficient (rho) for the associations between host age, host antibodies to IE (αIE), and expression of *var* genes of the infecting parasites in children aged 0–6, 0–12, and above 12 months. Red background shading indicate positive correlation while blue shading indicate a negative correlation. Increasing color intensity indicates increasing statistical significance of the correlation as indicated in the key [$p < 0.00005$ (darkest), $p < 0.05$ (lightest)]. **(A)** Associations of age of the children and their αIE levels with expression of cys2 and group A-like *var* genes, **(B)** associations of age of the children and their αIE levels with *var* transcript quantity obtained with primers targeting (i) group A *var* genes (gpA1, gpA2, dc13) and their proportion relative to the sum of the *var* transcript measured (gpA_prop), (ii)DC8 *var* genes (dc8-1, dc8-2, dc8-3, and dc8-4) and their proportional expression (dc8_prop), (iii) group B *var* genes (b1) and their proportional expression (b1_prop), and (iv) group C *var* genes (c2) and their proportional expression (c2_prop), **(C)** associations of age of the children and their αIE levels with *var* gene expression homogeneity (VEH). Also shown is the associations of parasitemia with age and αIE levels of the children and the relationship between αIE and age of the children. More details about the primers can be found in **Table S1**.

Var Expression Homogeneity (VEH) Was Negatively Associated With Host Age and Positively With Host Immunity (αIE) in the First Year of Life

Previously, we observed the homogeneity of *var* expression to increase with increasing host immunity. That is, the *var* gene expression in parasites from naïve children was more heterogeneous compared to semi-immune children such as asymptomatics (32). In this study, we observed, in the first year of life, VEH decreased with declining αIE and increasing age as demonstrated by the positive and negative association with host αIE and age, respectively (αIE; $p = 0.03$, age; $p = 0.001$, **Figure 1C**).

Parasitemia Increased With Declining αIE and Increasing Host Age in the First Year of Life

We finally examined the potential role of αIE in controlling parasitemia in the first year of life and after (**Figure 1C**). As shown in **Figure 1C**, parasitemia showed positive association with host age in the first 6 months of life ($p = 0.03$, **Figure 1C**). During this period, parasitemia also increased with declining

αIE as shown by the negative correlation with αIE ($p = 0.03$, **Figure 1C**). In contrast, parasitemia decreased with increasing host age and αIE after 12 months of life (age; $p = 0.002$, αIE; $p < 0.0001$, **Figure 1C**) when the children are expected to acquire αIE with time as a result of continuous exposure to malaria. This result reinforces a role for maternal αIE in limiting *in vivo* parasite expansion and severe malaria during infancy.

DISCUSSION

This study set out to investigate the relationship between the expression of *var* genes that have been associated with severe malaria and host age in children between 0 and 12 months, the age during which maternal immunity declines. We hypothesized that there will be a positive correlation between the expression of group A *var* genes and host age in children 0–12 months old after which the relationship will change to a negative correlation. As expected, DC8, DC13 and group A *var* genes showed positive associations with host age in children 0–12 months old.

The simplest explanation for these changes in *var* expression patterns is that they reflect the decay of maternal antibodies with increasing age in children between 0 and 12 months old, leading to increased survival of parasites expressing group A *var* genes. This allows parasites expressing group A *var* genes to dominate the infection. Subsequent acquisition of antibodies against these *var* genes results as children gain exposure to infection, consequently leading to a decline in group A-like *var* gene expression levels in older children (>12 months). Our result is consistent with the above explanation as αIE levels declined in the first 6 months of age, a period of rapid decline of maternal antibodies as observed in other studies (4, 5).

As expected the αIE showed negative association with both host age and *var* expression in the children between 0 and 12 months old and the subset <6 months of age. This contrasts with the positive relationship between host age and αIE in children older than 12 months (**Figure 1**). One caveat of the αIE data for the children 0–12 months is that the αIE levels were measured against one clinical isolate but in a previous study in which antibodies were tested against 8 different isolates, antibodies to each one of these correlated negatively with the expression of group A-like *var* genes suggesting that a single parasite isolate can be used as a surrogate of host immunity (18).

The explanation that the observed positive relationship between *var* gene expression and host age in the first year of life is due to the waning maternal antibodies is further supported by the *var* expression homogeneity data where significant negative and positive correlation with host age and αIE, respectively, was observed. That is, as the maternal antibody wanes, the *var* gene expression homogeneity decreases which is consistent with a previous study that demonstrated that homogeneity of *var* gene expression is lowest in children with severe malaria and low αIE as compared to children with asymptomatic infection and high αIE (32).

The observed rise in parasitemia with declining αIE during the first 6 months of life (**Figure 1C**) is also supportive of a role for maternal αIE in controlling parasitemia potentially through selection against parasites expressing group A and DC8 *var* genes. These subsets of genes mediate stronger cytoadhesion of IE and sequestration in the organs (13, 18, 20, 33), allowing IE escape removal by the spleen and thus promoting *in vivo* expansion of the parasite population. Maternal αIE targeting group A and DC8 *var* genes are expected to limit parasite expansion through inhibition of IE sequestration, rendering the IE vulnerable to splenic clearance (26, 34–36).

Other factors have been proposed to protect against malaria in infants and may play a role in modifying *var* expression during infancy. Fetal hemoglobin (HbF) has been demonstrated to decline in the first year of life (37). It is possible that the decline of HbF in the first year of life could explain the increasing expression of group A *var* genes over the same period and that this coincidentally mimics the effect of antibodies on parasite gene expression. Hemoglobinopathies such as sickle trait have been proposed to protect against malaria by reducing the amount of *Pf*EMP1 trafficked to the surface of IE (38). There is also evidence that sickle red cells can modify the parasite gene expression at transcriptional level through red cell microRNA that translocate into the parasite (39). This raises the possibility that other abnormal hemoglobin, such as HbF, may as well contribute to protection by reducing *var* gene expression and cytoadhesion (40). HbF has been suggested to work together with maternally acquired antibodies to protect infants from malaria (41) perhaps by impairing cytoadhesion and promoting clearance of IE. This suggestion is consistent with our result showing that expression of group A *var* genes increases over the period when both HbF and maternal antibodies are expected to wane. Dissecting the relative contribution of HbF and maternal αIE on the parasite *var* gene expression during the first year of life warrants further investigation.

In conclusion, we have tested the prediction that, if maternal antibodies play a role in protecting infants against severe malaria, they would impose a selection pressure on the infecting parasite population that is similar to that previously described in children as they gain exposure to *P. falciparum* infection. The results show that parasites infecting infants, show *var* gene expression patterns that are highly similar to those previously described for parasites under immune selection. Future studies that relate αIE levels in cord blood with *var* gene expression patterns during first infection, or longitudinal studies of mother-infant pairs, would be required to provide a definitive link between maternal antibodies and protection against severe malaria in the first months of life.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Harvard Dataverse, URL: https://doi.org/10.7910/DVN/VGRAVH.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by KEMRI/SERU/3149 Kenya Medical Research Institute. Written informed consent to participate in this study was provided by the participants' legal parents/guardians.

AUTHOR CONTRIBUTIONS

PB, KM, CK, and AA contributed to the design and implementation of the research. CK, AA, and MM performed the experiments with contributions from MH, GF, TO, GG, GW, and RR for sequence data and statistical analyses. The manuscript was prepared by CK with contributions from AA, KM, GF, and PB. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02328/full#supplementary-material

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Aryl Hydrocarbon Receptor-Signaling Regulates Early *Leishmania major*-Induced Cytokine Expression

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The early inflammatory skin micromilieu affects resistance in experimental infection with *Leishmania major*. We pursue the concept that macrophages, which take up parasites during early infection, exert decisive influence on the inflammatory micromilieu after infection. In order to analyze their distinctive potential, we identified differentially regulated genes of murine granuloma macrophages (GMΦ) from resistant and susceptible mice after their infection with metacyclic *Leishmania major.* We found induction of several cytokines in GMΦ from both strains and a stronger upregulation of the transcription factor aryl hydrocarbon receptor (AhR) in GMΦ from resistant mice. Using both an AhR agonist and antagonist we demonstrated that AhR is involved in *Leishmania*-induced production of TNF in macrophages. *In vivo*, single local injection of an AhR agonist in early lesions of susceptible mice caused an increased induction of *Tnf* and other cytokines in the skin. Importantly, local agonist treatment led to a reduction of disease severity, reduced parasite loads and a weaker Th2 response. Our results demonstrate that local activation of AhR has a beneficial effect in experimental leishmaniasis.

Keywords: skin infection, leishmaniasis, AhR, macrophages, epidermis

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INTRODUCTION

Interaction of pathogens and innate immune cells is a crucial early event in infection. It triggers immediate defense mechanisms and initiates pathogen-specific acquired immunity. Pathogens, however, have evolved mechanisms to circumvent activation of the immune system. Skin infection with intracellular parasite *Leishmania (L.) major* is an excellent murine model system to analyze host-pathogen interaction (1). Early after infection with *L. major*, macrophages take up infectious metacyclic promastigotes which then transform intracellularly into amastigotes. The innate immune system is unable to clear the infection without activation by T-cells. Ag-specific Th1 cells and their production of Interferon-γ (IFNγ) are necessary to achieve classical activation of macrophages and NO-mediated parasite clearing in resistant C57BL/6 mice (2). Susceptible BALB/c mice develop a Th2-response characterized by IL4 and IL13 secretion and fail to clear the parasite. Th-cell priming takes place in draining lymph nodes (dLN), but can be influenced by the inflammatory milieu of the infected skin (3–6). We already demonstrated differences in the early infiltrate of phagocytes with earlier appearance of mature macrophages in resistant mice (5, 7). Subsequently, we have identified several differentially regulated cytokines expressed in infected skin from resistant and susceptible mice early during infection. By altering the expression of some

of these cytokines in the early inflammatory skin milieu, e.g., IL-6 and CXCL11, we were able to significantly influence polarization of Th-cells (5, 6).

Macrophages are among the first cells to interact with parasites and are capable of sustained production of inflammatory mediators. We thus consider them a prime candidate to determine the early inflammatory micromilieu.

Infection of macrophages with *L. major* has been shown to induce cytokine secretion, upregulation of genes and activation of cellular signaling pathways like ERK1/2, p38MAPK and NF-kappa-B (8–12). On the other hand, suppression of gene expression and inhibition of NF-kappa-B-signaling were reported (10, 13). For example, *L. major* uptake by macrophages abrogates LPS-induced IL12 secretion (8, 14).

However, differential reaction of macrophages to *L. major* also depends on the origin or activation of investigated macrophages or subtype of monocytes (15–18).

L. major-induced IL12 suppression was more pronounced in peritoneal macrophages compared to bone marrow macrophages (19). Similarly, a comparison of inflammatory and resident peritoneal macrophages infected with *L. major* revealed that resident, but not inflammatory macrophages induced production of various cytokines and reactive oxygen species (20).

Therefore, since macrophage subtypes can be different in response to *L. major,* we wanted to analyze cells which most closely resemble skin macrophages. Such cutaneous macrophages, well-suited for our study, can be isolated in considerable numbers from non-immune polyacrylamide granulomas (8, 21).

Strain-specific differences in skin macrophages could affect the early inflammatory milieu. Belkaid et al. already compared IL12, IL6, and TNF secretion of granuloma-macrophages (GMΦ) from C57BL/6 and BALB/c mice infected with *L. major*, and found no differences. For a more global analysis however, we performed microarray analysis of GMΦ infected with metacyclic *L. major* promastigotes. We identified differentially regulated genes between infected GMΦ from BALB/c and C57BL/6 mice. Among those we found transcription factor aryl hydrocarbon receptor (AhR), an inductor of xenobiotic compound-degrading enzymes, deserving a closer look because of its expanding role in immunity, especially macrophage function (22–26). Most relevant to our setting, Climaco-Arvizu et al. found a role for AhR in the regulation of nitric oxide and arginase production in mouse macrophages. Peritoneal macrophages from *Ahr* knockout mice showed reduced NO production, but enhanced secretion of several cytokines like TNF and IL12 when polarized to a M1 subtype and stimulated with LPS (26). *Ahr* knockout macrophages were more susceptible to *in vitro Leishmania* infection. In a previous work, the same group also reported that *Ahr* knockout mice on a resistant background showed an exacerbated immune response characterized by lower numbers of regulatory T-cells that, while causing more severe inflammation at first, ultimately resulted in accelerated control of the parasite (24). As AhR is a transcriptional regulator implicated in resistance against *Leishmania* both *in vitro* in macrophages and *in vivo*, we chose to further analyze the role of AhR in early events determining resistance against *L. major*.

MATERIALS AND METHODS

Experimental Leishmaniasis

Specific pathogen free mice from C57BL/6 and BALB/c strains were bred in the animal facility of the Department of Dermatology, Münster, or obtained from Charles River, Germany, and used at 8-12w of age. All experiments were approved according to the animal welfare laws of the Federal Republic of Germany by the animal welfare authority of the state of North Rhine- Westphalia, filed under reference 84-02.04.2015.A348.

Leishmania parasites of strain MHOM/IL/81/FE/BNI were grown in Schneider's *Drosophila* medium supplemented with 10% fetal calf serum (FCS), 2% human urine, 2% glutamine, and 1% penicillin-streptomycin at 25°C and 5% CO_2.

Male BALB/c mice (5 per group) were infected in the left hind foot with 2×10^7 stationary phase parasites in 20 µl PBS to allow a comparison to our previously published data on the early phase of experimental leishmaniasis (5, 6). Mice were sacrificed after 20 h for measurement of epidermal gene expression or after 11 d (early Th response), and 4 or 5 w for infection experiments (late Th response, parasite load). Foot swelling was measured weekly using a caliper with the uninfected foot serving as control. All experiments were repeated at least 5 times.

For simultaneous treatment with AhR ligands, 30 nmol of agonist ITE (2-(1*H*-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester, Tocris Bioscience) in 1 µl DMSO or solvent control was included in the final volume.

Comparison of parasite dissemination was performed by limiting dilution assay of infected dLN using *Leishmania* growth medium as described previously (5, 18) by using Leishmania medium instead of slant blood agar. Briefly, foot skin and dLN were removed aseptically and homogenized in 5 ml Leishmania medium. Serial dilutions were carried out in quadruples (100 µl culture volume each) using 96-well tissue plates. After culture for 1 w, the highest dilution yielding growth of viable parasites was determined using a phase contrast microscope.

Cytokine Assay

Cytokines from restimulated *L. major*-specific dLN cells were assessed using a mixed lymphocyte reaction as previously described (5). Briefly, the dLN from infected animals were mashed through a cell strainer in PBS, washed and transferred to uncoated 96-well U-bottom plates in RPMI 1640 containing 2 mM glutamine, 50 µM mercaptoethanol, and 10% FCS at 2×10^6 cells/well. Cells were restimulated with 1 µl soluble Leishmania antigen prepared by repeated freeze/thaw cycling of 5×10^8 stationary phase parasites in 1 ml PBS. After 5d at 37°C and 5% CO_2, IFNγ and IL4 secretion were measured by cytometric bead assay using FlexSets by BD Bioscience (San Jose, California) according to the manufacturer's protocol.

Granuloma Macrophages

GMΦ were recovered from polyacrylamide gel pouches as described in John et al. (21). In short, sterile polyacrylamide gel (BioGel P-100, Bio-Rad, Germany) was injected subcutaneously in two 1 ml portions on the back of the animal. The gel was

recovered after 48 h, resuspended in PBS and given through a cell strainer and then washed. GMΦ were then left to adhere in petri dishes with 10 ml DMEM medium containing 20% L-929 cell (ATCC #CCL-1) supernatant and 10% FCS at 1×10^6cells/ml for 24 h at 37°C and 7% CO_2. Cells were incubated with 10 mM EDTA in PBS for 10 min at 37° and resuspended by repeated up and down-pipetting. Cells were then washed with PBS and resuspended in DMEM containing L-929-cell supernatant at 1×10^6cells/ml and left to adhere overnight in 12-well plates. Metacyclic *L. major* promastigotes were prepared from stationary cultures. Briefly, stationary phase cultures are centrifuged several times to enrich metacyclic parasites in the supernatant by their density after which the remaining parasites are collected and run over a Ficoll gradient as described in more detail by Späth et al. (27). Macrophages were incubated with a 5 × MOI of metacyclic *L. major*. 1 µl of 30 mM solutions of AhR antagonist CH-223191 and agonist ITE in sterile DMSO per ml medium were added where indicated, with untreated groups receiving 1 µl of DMSO. TNF concentration of the supernatant was measured using the cytometric bead assay FlexSet for murine TNF by BD Bioscience (San Jose, California). Parasite phagocytosis was measured by FACS analysis of cells incubated with a 5 × MOI of fluorescein isothiocyanate (FITC) -stained parasites in the presence of DMSO, ITE and CH-223191 using the same medium and concentrations as above. The FITC-staining was performed as described earlier (18). For assessment of parasite killing, cells where then washed and incubated in the presence of 500U of rmIFNγ (PromoCell, Germany) for another 20 h. Production of NO was measured by photometric measurement of nitrite in supernatant at 560 nm using Griess reagent and a nitrite standard curve after 24 h preincubation of 5×10^5 cells/ml with 500U of recombinant murine IFNγ) followed by further 24 h incubation with *L. major* in the presence of 500U/ml rmIFNγ as described in Ehrchen et al. (18).

Human Blood Monocytes

Human monocytes were isolated from fresh human blood leukocyte reduction chambers of platelet apheresis sets from healthy, voluntary whole blood donations after informed consent of the donors according to the regulations of the blood bank of the University Hospital Münster by Pancoll (PAA Laboratories, Austria) and subsequent Percoll (GE Healthcare) gradient centrifugation as described previously (28). Purity of monocytes was > 85%, as assessed by staining with CD14 antibody (Becton Dickinson) and FACS analysis. Cells were cultivated in McCoy's 5a medium supplemented with 15% FCS, 1% l-glutamine and 1% non-essential amino acids (all from Biochrome, Germany) and without antibiotics in uncoated 12 well-plates, and were allowed to rest overnight prior to experiments (37°C, 7% CO2). Cells were infected with a 5 × MOI of stationary phase *L. major*. Because of the reported lower affinity of the human AhR compared to murine AhR (29), we added 10 µl of 30 mM solutions of AhR ligands or carrier per ml of medium.

Real-Time PCR and Microarray Analysis

RNA from GMΦ and human monocytes was extracted using the Quiagen RNeasy micro kit according to the manufacturer's instructions. For RNA extraction from mouse feet, skin was homogenated in RNeasy lysis buffer using a peqlab Precellys homogenizer for a single run at maximum power and time settings. Semi- quantitative RT-PCR was performed as described previously on a Bio-Rad CFX384 Touch Real-Time PCR detection system (28).

For microarray analysis, total RNA from three independent experiments of 4 h *L. major* infected C57BL/6 or BALB/c GMΦ was isolated and subsequently processed for microarray hybridization using Affymetrix Murine Genome MG_U74Av2 arrays according to the manufacturer's instructions (Affymetrix). Arrays were developed and analyzed as previously described (5). Microarray data were analyzed using MicroArray Suite Software 5.0 (Affymetrix) using data from corresponding control samples as baseline.

We retained only genes which were significantly regulated in every single experiment (change p-value < 0.05, fold-change $\geq$1.5, expression over background) as well as in the complete set of experiments (fold-change of $\geq$1.5, p-value of $<$0.05, paired t-test).

To compare *L. major* induced alterations expression patterns between macrophages isolated from resistant and susceptible mouse strains, signal log ratios of infected vs. uninfected control samples in both mice strains were evaluate by paired t-test. We retained only genes with a $p < 0.05$ and a differential fold-change regulation of $\geq$1.5.

To identify transcription factors with statistically over-represented binding sites in promoter regions of regulated genes, we used CARRIE with the implemented promoter sequence analysis tool ROVER (30). Promoter sequences were defined 1000 bases upstream to 100 bases downstream of the transcription start site and obtained using PromoSer (31) and equally sized group of control genes with stable expression but no detectable regulation by *L. major* infection were compared.

RESULTS

Transcriptional Changes in *L. major* Infected GMΦ

To identify genes that are regulated during infection of macrophages with *L. major*, GMΦ from C57BL/6 and BALB/c mice where incubated with metacyclic *L. major* for 4 h with a multiplicity of infection (MOI) of 5:1. More than 75% of macrophages had taken up *L. major* parasites at this timepoint. Using a cut-off value of 1.5-fold, 141 genes were significantly upregulated and 127 were significantly downregulated in GMΦ from C57BL/6 mice, whereas 69 genes were upregulated and 91 where downregulated in GMΦ from BALB/c mice. Among upregulated genes we found *Tnf* and other cytokine genes, transcription factors, genes associated with apoptosis, lipid metabolism and the NF-kappa B cascade. **Table 1** shows a selection of upregulated genes. Array data was uploaded to the NCBI GEO repository under accession number GSE127541. **Supplemental Tables 1**, **2** contain a complete list of regulated genes in C57BL/6 and BALB/c mice, respectively.

TABLE 1 | Genes significantly upregulated by *Leishmania major* infection.

Gene Symbol	Description (NCBI gene)	*n*-fold C57BL/6	*n*-fold BALB/c	*p*-value for differential regulation in C57BL/6 vs. BALB/C
Chemokines and receptors				
Ccl4	Chemokine (C-C motif) ligand 4	24.9	8.7	n.s
Cxcl2	Chemokine (C-X-C motif) ligand 2	21.0	12.0	n.s.
Cxcl1	Chemokine (C-X-C motif) ligand 1	17.9	10.7	n.s.
Ccrl2	Chemokine (C-C motif) receptor-like 2	2.6	3.2	n.s.
Cytokines and related molecules				
Tnf	Tumor necrosis factor	18.2	8.2	n.s.
Il1a	Interleukin 1 alpha	10.7	6.1	n.s.
Il1rn	Interleukin 1 receptor antagonist	5.1	3.2	n.s.
Apoptosis				
Myc	Myelocytomatosis oncogene	2.7	−0.2	0.007
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	4.3	2.8	n.s.
Socs3	Suppressor of cytokine signaling 3	3.9	2.3	n.s
Casp4	Caspase 4, apoptosis-related cysteine protease	2.3	1.7	n.s.
Sod2	Superoxide dismutase 2, mitochondrial	2.3	1.6	n.s.
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	1.9	1.6	n.s.
Receptors and cell surface proteins				
Vcam1	Vascular cell adhesion molecule 1	10.6	3.0	n.s.
Olr1	Oxidized low density lipoprotein (lectin-like) receptor 1	9.0	2.5	<0.001
Icam1	Intercellular adhesion molecule	3.3	1.7	n.s.
Adora2a	Adenosine A2a receptor	3.2	2.2	n.s.
Mapkkk cascade				
Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	3.1	2.8	n.s.
Cav	Caveolin, caveolae protein	2.3	0.7	0.008
Mapkapk2	MAP kinase-activated protein kinase 2	2.0	1.5	n.s.
Dusp1	Dual specificity phosphatase 1	2.1	1.5	n.s.
Other genes involved in immune response				
Tnip1	TNFAIP3 interacting protein 1	2.5	1.7	n.s.
Traf5	Tnf receptor-associated factor 5	2.3	1.6	n.s.
Slfn2	Schlafen 2	3.1	0.7	0.034
Mmp13	Matrix metalloproteinase 13	2.5	1.6	0.031
Ifi205	Interferon activated gene 205	2.2	−1.1	0.001
Traf1	Tnf receptor-associated factor 1	12.8	8.1	0.049
Lipid metabolism				
Ptgs2	Prostaglandin-endoperoxide synthase 2, cox-2	47.2	8.8	n.s.
Ptges	Prostaglandin E synthase	3.8	3.0	n.s.
Transcription factors				
Fosl1	Fos-like antigen 1	14.9	4.2	n.s.
Ahr	Aryl-hydrocarbon receptor	8.7	4.6	0.020
Spic	Spi-C transcription factor (Spi-1/PU.1 related)	7.4	4.4	n.s.
Protein import into nucleus/nf-kappa b cascade				
Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	3.8	2.7	n.s.
Nfkbib	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta	2.2	1.7	n.s.
Kpna3	Karyopherin (importin) alpha 3	2.0	1.8	n.s
Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	2.3	1.8	n.s
Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	2.3	1.8	n.s

TABLE 2 | Top ten overrepresented transcription factor binding sites among genes upregulated in *Leishmania major*-infected C57BL/6 granuloma macrophages.

Binding site	*p*-value
NF-kappaB binding site	3.14E-08
Myogenic enhancer factor 2	0.00000382
Cellular and viral TATA box elements	0.00000391
Signal transducer and activator of transcription 1	0.00000634
Paired box factor 2	0.00000805
c-Rel	0.000148
ATF-1	0.000159
AhR	0.000372
BTB and CNC homolog 1	0.000526
HNF-3/Fkh homolog-8	0.000621

Infected GMΦ From Resistant Mice Upregulate *Ahr*

While most genes were similarly regulated in resistant and susceptible mice, we found only eight genes that showed strain-specific differences. Among them was the transcription factor *Ahr*.

To check whether AhR-signaling could be involved in the underlying transcriptional regulatory networks, we analyzed overrepresentation of transcription factor binding sites among regulated genes using CARRIE analysis, and found AhR binding sites overrepresented in upregulated C57BL/6 genes with a *p*-value of 0.000372 (**Table 2**).

Therefore, and because AhR has recently been identified as an important regulator of innate immunity (22, 26, 32–34) and was implicated in regulation of resistance to experimental leishmaniasis (24, 35, 36), we focused on AhR.

We first confirmed gene regulation of *Ahr* in GMΦ using *RT-PCR*. In agreement with microarray data, uptake of *L. major* induced *Ahr* expression significantly in both mice strains. *Ahr*

induction was significantly stronger in C57BL/6 vs. BALB/c mice (**Figure 1A**). The strain specific difference in AhR upregulation between GMΦ from resistant and susceptible mice indicated a possible beneficial role of AhR in resistance to *Leishmania*.

AhR Ligands Change Cytokine Secretion by *L. major* Infected Macrophages

Since AhR itself presents a transcription factor and AhR binding sites were overrepresented among *L. major*-induced genes, we checked for relevance of AhR in *Leishmania*-induced gene expression. To this end we analyzed gene expression of C57BL/6 derived GMΦ after treatment with AhR antagonist CH-223191. We observed a reduction in *Tnf* expression (**Figure 1B**). Conversely, treatment with the AhR agonist (2-(1*H*-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE) caused a higher expression of *Tnf* in macrophages co-incubated with *L. major* for 4 h (**Figure 1C**). Subsequently, we analyzed the effects of AhR antagonist CH-223191 (**Figure 1D**) or AhR agonist ITE (**Figure 1E**) on production and release of TNF protein by infected GMΦ. As shown previously using intracellular FACS analysis in GMΦ (8), GMΦ secreted TNF protein upon *L. major* infection. As indicated by RT-PCR data we found reduced secretion of TNF protein by cells treated with AhR antagonist, while those treated with AhR agonist showed enhanced secretion. In terms of general macrophage function, FACS analysis with FITC-stained parasites indicates that most cells contain parasites after 4 h, regardless of AhR ligand treatment. Macrophages are usually not able to effectively eliminate parasites since *L. major* inhibits upregulation of inducible nitic oxide synthase necessary for parasite killing (37). Also, since it has been observed that *Ahr* knockout peritoneal macrophages produce less NO upon LPS/IFNγ stimulation (26), we analyzed parasite killing and NO production in *L. major*/IFNγ stimulated cells. However, incubation for 20 h in the presence of IFNγ revealed no obvious differences in killing between cells treated with agonist or antagonist and untreated cells (**Figure 2A**; **Supplemental Figure 1**). Similarly, *Leishmania*-induced NO production in IFNγ-stimulated cells was unaffected by treatment with either agonist or antagonist (**Figure 2B**).

L. major Infected Human Blood Monocytes Do Not Upregulate *Ahr* but AhR Antagonist Treatment Reduces *Tnf* Expression

We also treated human monocytes co-incubated with *Leishmania* parasites with AhR agonist and antagonist in order to assess the translational potential of our findings in murine granuloma macrophages. We could not detect upregulation of *Ahr* in human monocytes from healthy donors in response to *in vitro Leishmania* infection (**Figure 3A**). While *Tnf* expression was induced in infected monocytes after 4 h, there was no additional effect of treatment with AhR agonist ITE (**Figure 3B**). However, we could observe a reduced induction of *Tnf* expression in human monocytes treated with AhR antagonist CH-223191 (**Figure 3C**). When measuring TNF secretion after 24 h using ELISA, variability was very high between samples and there were no differences reaching statistical significance. As the kinetics might be different between these cell types, we measured TNF secretion after 4 h and found no difference with agonist treatment (**Figure 3D**). When treated with AhR antagonist however, there was a trend toward reduced TNF secretion (**Figure 3E**), but it failed to reach our significance threshold with $p = 0.06$.

Treatment of Infected Mice With AhR Ligands Influences the Cytokine Milieu *in vivo*

Having shown that *Ahr* is involved in *L. major*-induced gene expression by macrophages and that it increases release of TNF, we addressed our concept that gene expression in macrophages is decisively involved in generating a resistance-inducing early cutaneous micromilieu *in vivo*. Since we had previously demonstrated that *L. major* infection resulted in an induction of cytokines in infected skin (5), we analyzed whether interference with AhR-signaling modulates induction of these cytokines. Therefore, we injected BALB/c mice with AhR agonist ITE during infection with *L. major* and measured expression of genes using *RT-PCR* of foot skin samples after 20 h. We observed a significant increase in expression of *Tnf, Cox2 Cxcl2* and *Cxcl10* (**Figures 4A–D**). We demonstrated AhR-dependent induction of both well-established (*Cox2*), but also novel AhR target genes (*Cxcl10*) in *L. major*-infected skin, while expression of other genes (*Il12, Il10, Il4, Il6, Il1β, Ccxcl11, Il1a*, and *Cxcl1*) was not influenced (data not shown). Thus, signaling by AhR selectively induces genes which are known to be markedly present in the early cutaneous micromilieu after *L. major* infection.

Treatment of Infected Mice With AhR Ligands Increases Resistance to *L. major* Infection in BALB/c Mice

Since increased *L. major*-induced gene-expression in C57BL/6 mice in comparison to BALB/c mice is associated with resistance (5) and AhR-induced TNF is one known decisive agent in leishmaniasis (38–41), we wondered if susceptible mice could benefit from local AhR activation during early leishmaniasis. Therefore, we treated susceptible BALB/c mice with AhR agonist ITE, given as a single injection at the time of infection, reflecting its rapid induction in GMΦ of resistant mice. We observed a rapid increase in footpad swelling typical for high dose infection with this *L. major* strain. Importantly, we observed a small, but significant reduction in foot swelling during the first weeks of infection (**Figure 5A**), and, more relevantly, also more than 4-fold reduced parasite loads in dLN after 4w of infection (**Figure 5B**). We did not detect differences in parasite loads earlier during infection (11d, not shown). However, we were able to demonstrate reduced secretion of Th2 cytokine IL4 by popliteal lymph node cells restimulated with soluble *Leishmania* antigen (SLA), both early after parasite inoculation (11d, **Figure 5C**) and in established infection (4w, **Figure 5D**). Secretion of Th1 cytokine IFNγ was increased early after parasite inoculation, but this difference did not reach statistical significance and could not be observed in established

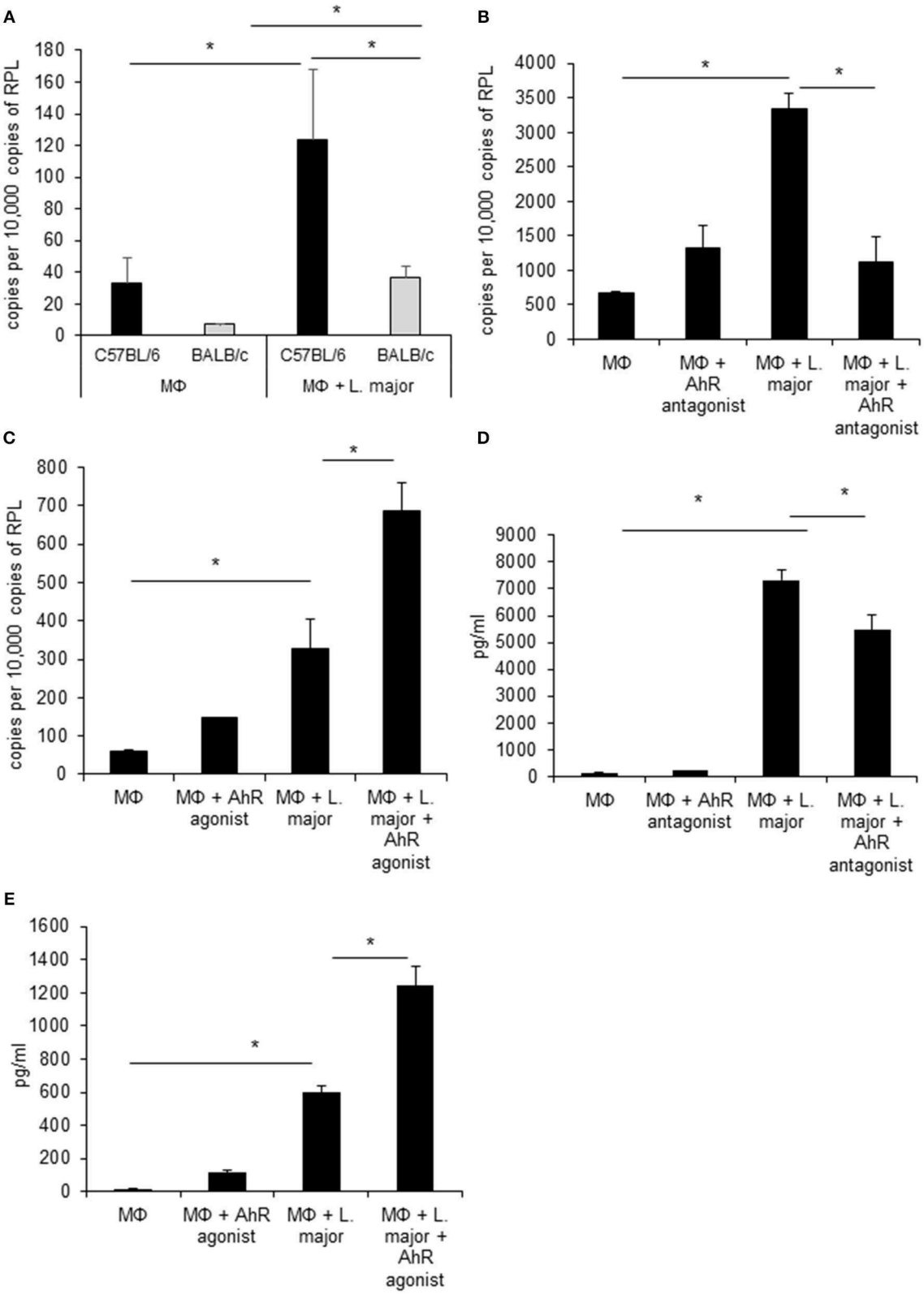

FIGURE 1 | Infected granuloma macrophages express AhR upon infection with *L. major*, which regulates *Tnf*α expression and secretion. RT-PCR data showing differential induction of AhR in granuloma macrophages from C57BL/6 (black bars) and BALB/c mice (grey bars) infected with a 5 × MOI of metacyclic *L. major* for 24h

(Continued)

FIGURE 1 | (A). RT-PCR of *Tnf* mRNA expression from C57BL/6 macrophages treated with AhR antagonist CH-223191 **(B)** and AhR agonist ITE **(C)** after incubation with a 5 × MOI of metacyclic *L. major* for 4 h **(B)**. TNF secretion by macrophages from C57BL/6 mice infected with *L. major* for 24 h in the presence of 30 nmol/ml AhR antagonist CH-223191 **(D)** and TNF secretion by macrophages infected with *L. major* for 24 h in the presence of 30 nmol/ml AhR agonist ITE **(E)**. $^*p < 0.05$, $n = 3$. Data shown is representative of three independent experiments with similar results.

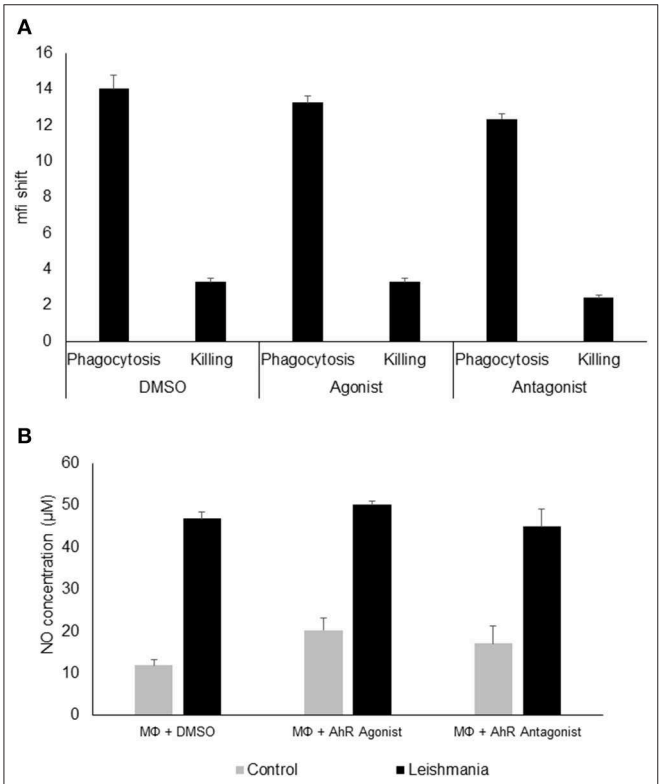

FIGURE 2 | AhR ligands do not affect phagocytosis, killing or NO production in granuloma macrophages. FACS analysis of granuloma macrophages incubated with a 5 × MOI of FITC-stained metacyclic *L. major* parasites in the presence of DMSO (carrier control), AhR agonist ITE or AhR antagonist CH-223191 for 4 h (phagocytosis) and after washing and further 20 h incubation in the presence of 500 units rmIFNγ (killing). Graphs display the mean fluorescence intensity shift in the FITC channel gated on macrophages incubated with stained parasites as compared to uninfected controls **(A)**. Measurement of NO production of macrophages prestimulated with 500U of rmIFNγ for 1 d followed by infection with 5 × MOI of *L. major* for 24 h treated with AhR agonist ITE, AhR antagonist CH-223191 and carrier control (DMSO) in the presence of 500U of rmIFNγ **(B)**, $n = 3$.

infection (**Figures 5E,F**). This indicates that the Th2 response in AhR agonist-treated BALB/c mice is diminished, albeit not completely switched toward a Th1 response. In agreement with this, both the differences in foot swelling and parasite loads diminished during prolonged infection after 4 weeks (data not shown). To ascertain the transient nature of AhR agonist single treatment- induced differences in lesional *Tnf* expression, we also measured *Tnf* expression in the skin after 3w of infection. While there is residual *Tnf* expression in the lesion at this time point, we found no difference between treated and untreated animals (**Figure 6**).

DISCUSSION

In order to identify how macrophages contribute to the early cutaneous cytokine milieu, we analyzed *L. major*-induced gene expression profiles of tissue macrophages from resistant and susceptible mice. Using this experimental approach, we demonstrated that *L. major* infection caused a marked induction of several immune modulatory genes in tissue macrophages from both mice strains. Different macrophage subtypes respond differently to *L. major* and so far, *L. major*-induced gene expression profiles were analyzed using bone marrow-derived or peritoneal macrophages. In contrast to data from bone marrow macrophages infected for 24 h (13), we did not detect a general suppression of gene expression, but the number of repressed and induced genes was rather similar after 4 h of infection. This supports the observation that a dominant suppression of macrophage gene expression occurs only during prolonged infection (13).

Upregulation of several inflammatory cytokines has been seen as part of a global gene expression response in bone marrow-derived macrophages from resistant and susceptible strains infected with a Tunisian isolate of *L. major* (42). Their *L. major*-induced gene expression profile was otherwise distinct from our results. These differences probably reflect the heterogeneity of different macrophage and parasite populations and stress the importance of clarifying the physiological significance of *in vitro* data using *in vivo* models.

In our *L. major*-induced gene expression profile we found that most genes were similarly regulated in resistant and susceptible mice (**Table 1**). Only eight genes showed strain-specific differences, among them the transcription factor *Ahr,* which was more strongly upregulated in macrophages from resistant animals. AhR is emerging as an important regulator in host defense and homeostasis [for a review, see (43)]. It was also recently described as a regulator in macrophage polarization (26) and several publications have reported a role for AhR in resistance to experimental Leishmaniasis (24, 35, 36).

Since *Ahr* expression was induced, and since we also found a significant overrepresentation of AhR binding sites among promoter regions of genes upregulated by *L. major* infection, we expected that AhR could be involved in regulating *L. major*-induced gene expression in macrophages. Both positive and negative interaction with important inflammatory transcription factors like the Nf-kappa-B family in myeloid cells have been described for AhR (44). AhR activity has been shown to attenuate macrophage cytokine production in response to LPS (25, 32, 34). Similarly, peritoneal macrophages from *Ahr* knockout mice produced higher amounts of TNF and other cytokines in response to IFNγ and LPS and showed lower expression of M2 markers upon IL4 stimulation (26). Importantly, while TNF is induced in LPS-stimulated *Ahr* knockout macrophages,

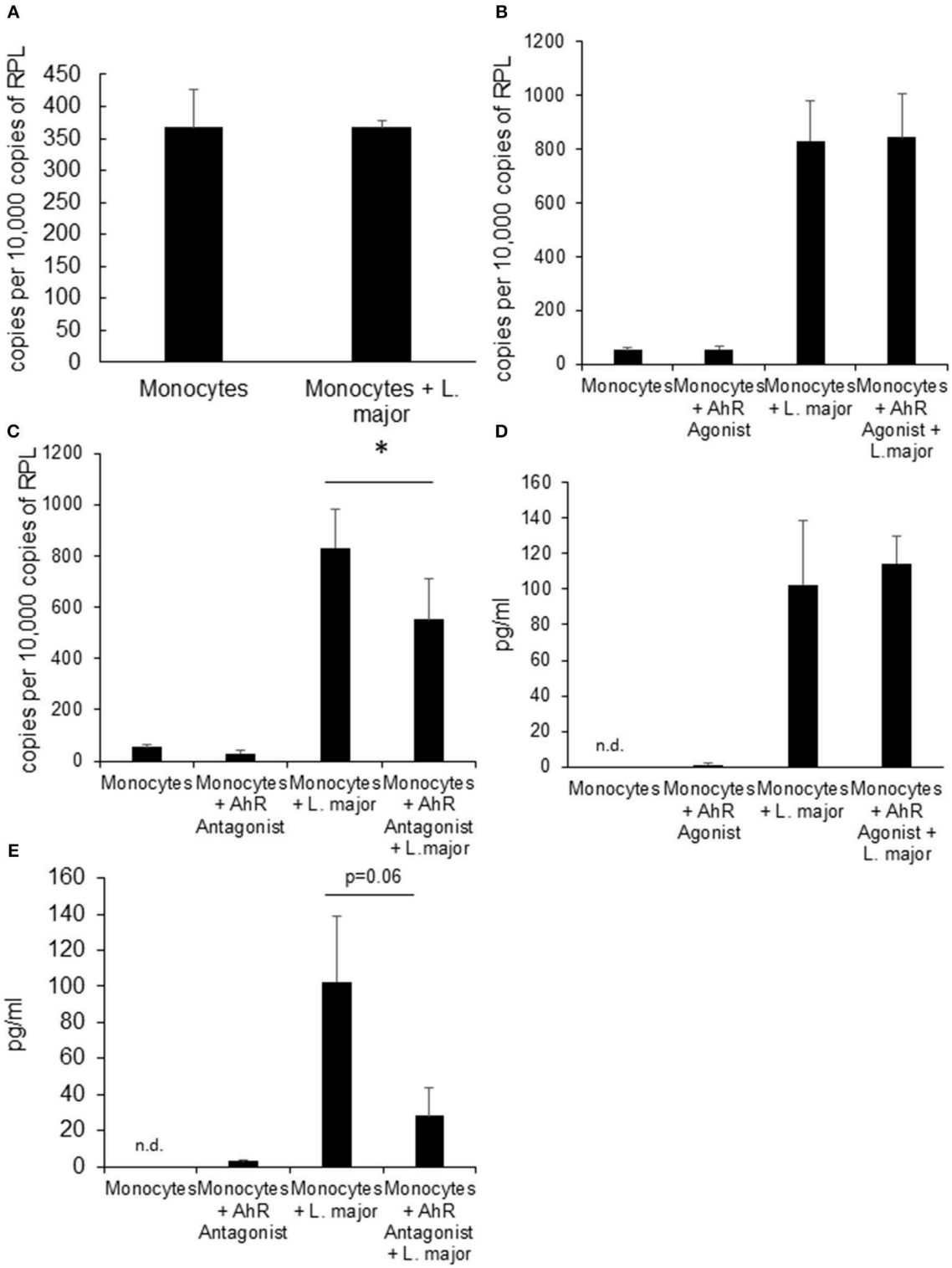

FIGURE 3 | Human monocytes do not upregulate *Ahr* upon infection with *L. major* but treatment with AhR antagonist reduces *Tnf* expression. RT-PCR data showing no induction of *Ahr* in human blood monocytes infected with a 5 × MOI of metacyclic *L. major* for 24 h **(A)**. RT PCR of *Tnf* mRNA expression from human monocytes treated with AhR agonist ITE **(B)** and antagonist CH223191 **(C)** after incubation with 5 × MOI of metacyclic *L. major* for 4 h. TNF concentration in the supernatant of human blood monocytes infected with a 5 × MOI after 4 h of infection in the presence of AhR agonist ITE **(E)** and antagonist CH223191 **(D)**. The graphs represent cumulative data from 3 independent experiments from individual blood donors. $^*p < 0.05$.

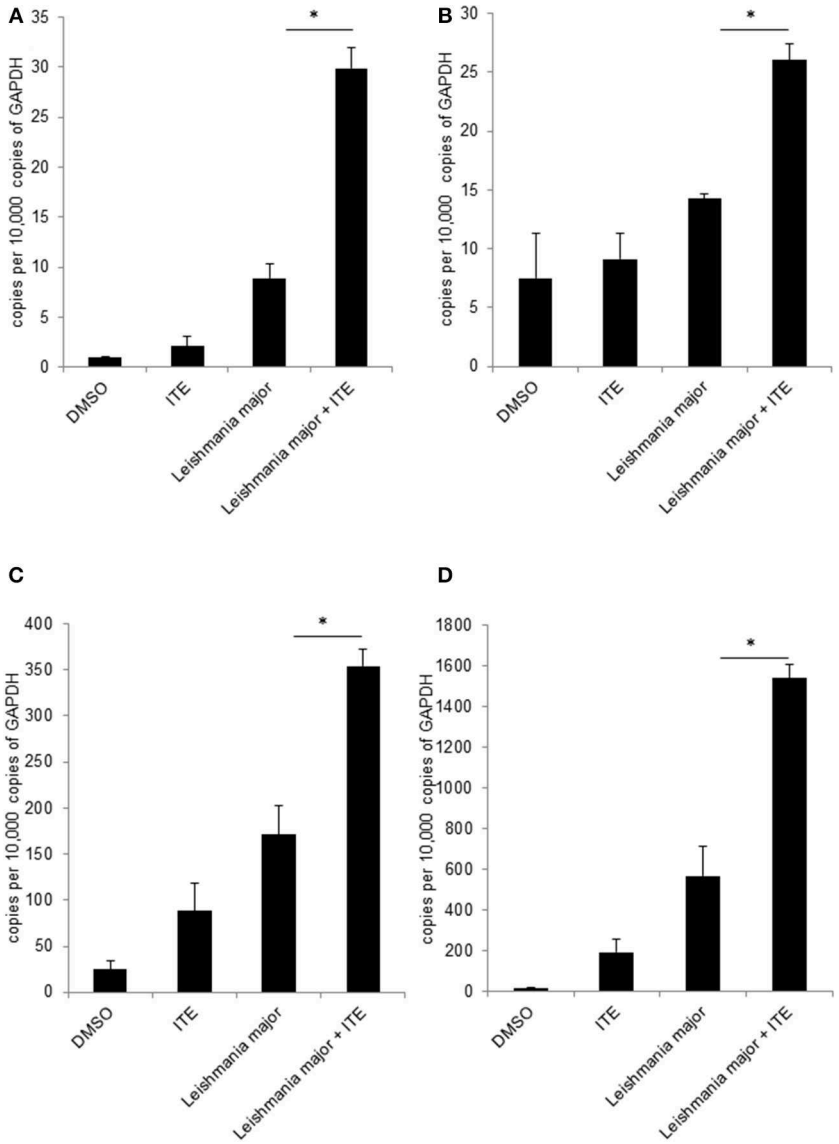

FIGURE 4 | Early local AhR treatment changes the local cytokine milieu. Susceptible BALB/c mice were treated with 1 μl of 30 mM (30 nmol) solution of AhR agonist ITE during inoculation with 2×10^7 *L. major*. Skin samples were taken after 20 h of infection for RT-PCR analysis. We show expression levels of *Tnf* **(A)**, *Cox2* **(B)**, *Cxcl2* **(C)** and *Cxcl10* **(D)**. $^*p < 0.05$, $n = 3$. Data shown is representative of three independent experiments with similar results.

production of NO is inhibited, indicating an ambiguous nature of AhR-signaling in terms of macrophage functions relevant for Leishmaniasis. There are also reports that AhR ligand TCDD induces TNF production in the human macrophage cell line THP1 via the AhR pathway (45), underlining the complexity of the relationship of AhR activity with TNF production.

We now demonstrate that activation of AhR using non-persistent pharmacological AhR agonist ITE enhances TNF production in GMΦ, while treatment with AhR antagonist CH-223191 diminishes TNF production during *L. major* infection. This is seemingly contradictory to the upregulation of TNF observed in IFNγ/LPS- stimulated macrophages from *Ahr* knockout mice, which would suggest a suppressive role of AhR in macrophage TNF secretion (26), but as mentioned above, TCDD induces TNF in human THP1 cells via activation of AhR (45), so whether AhR activity induces or suppresses TNF might well-depend on the specific macrophage type, activation status and stimulus. The observation that *Ahr* knockout peritoneal macrophages produce less NO upon LPS/IFNγ stimulation (26) indicates that AhR activity enhances NO production in macrophages. However, in our experiments, IFNγ- activated granuloma macrophages treated with AhR ligands did not exhibit changes in NO secretion upon infection with *L. maj*or. The lack of difference in NO production was accompanied by a lack of difference in parasite load of infected, IFNγ-activated

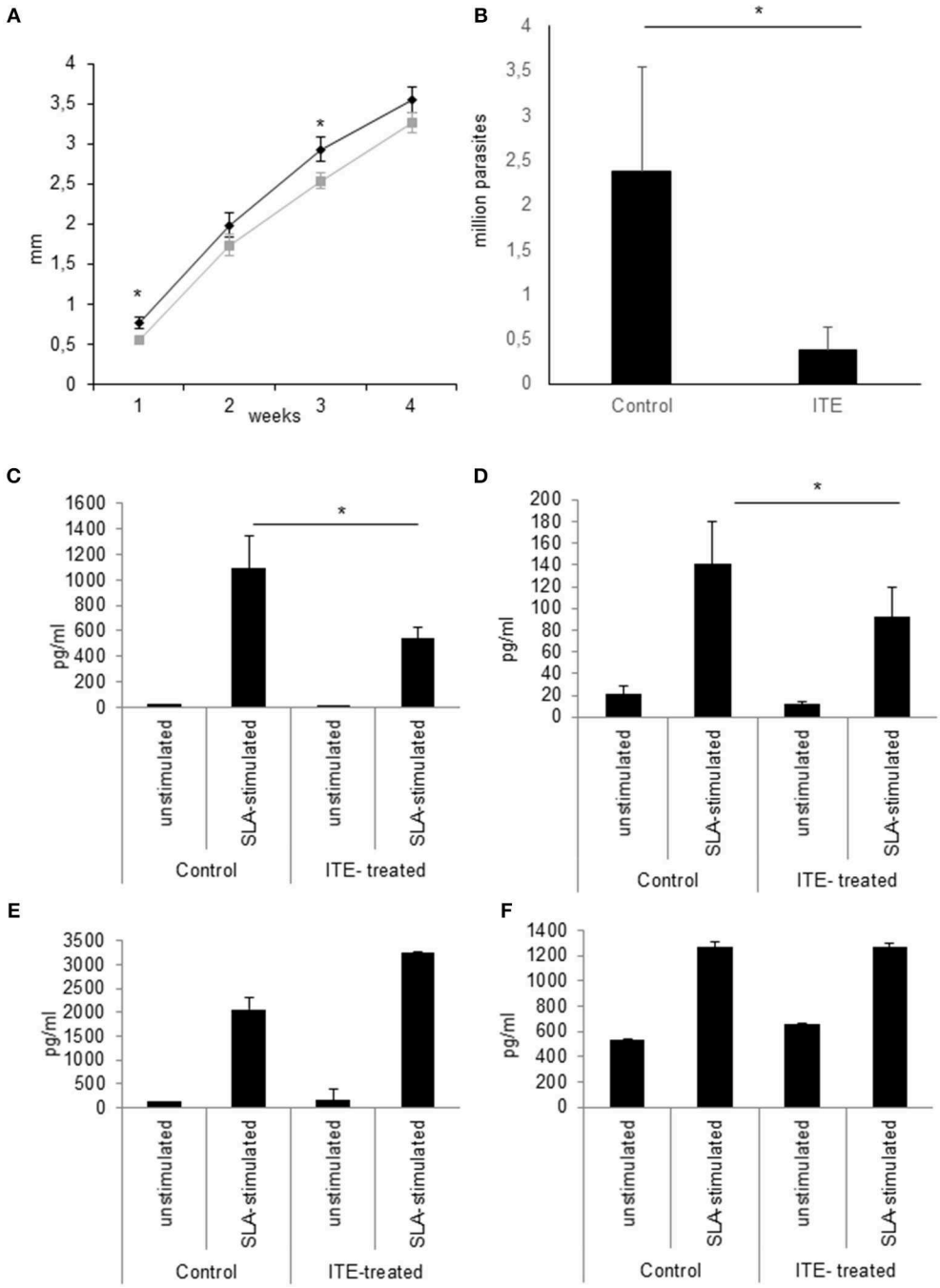

FIGURE 5 | Early local AhR treatment reduces infection severity in susceptible animals Treatment of susceptible BALB/c mice ($n = 5$ per group) with 30 nmol of AhR agonist ITE during inoculation with 2×10^7 *L. major*. Course of the infection was monitored by measuring the foot swelling in mm **(A)**. Differences in parasite dissemination were measured by limiting dilution assay of popliteal lymph node cells after 4w, $n = 5$ **(B)**. The quality of the T-cell response was assessed by measuring the secretion of Th2 cytokine IL4 by popliteal draining lymph node cells restimulated with soluble *Leishmania* antigen for 5d after 11d **(C)** and at w4 experiments **(D)**. Th1 cytokine IFNγ was measured accordingly **(E,F)**. $^*p < 0.05$, $n = 5$. Data shown is representative of 5 independent experiments with similar results.

macrophages treated with either ligand. These differences in macrophage responses might again reflect the heterogeneity of macrophage subtypes and response patterns to different stimuli (46).

We also tested the role of AhR-signaling in human monocytes. *Ahr* mRNA was not upregulated in human blood monocytes upon infection but AhR antagonist significantly reduced expression of *TNF* mRNA while agonist treatment had no effect.

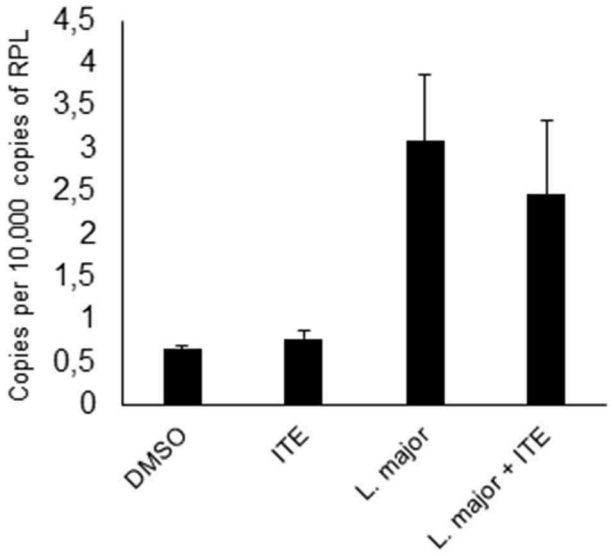

FIGURE 6 | Early local AhR agonist- treatment induced differences in *Tnf* expression are transient. Susceptible BALB/c mice were treated with 1 μl of 30 mM (30 nmol) solution of AhR agonist ITE during inoculation with 2×10^7 *Leishmania major*. Skin samples were taken after 3w of infection for RT-PCR analysis. $n = 5$.

Thus, the effects were not analogous to the response of murine granuloma macrophages but the reduction of *TNF*-mRNA nevertheless indicates a possible role of AhR-signaling in human *L. major* infected monocytes. These data are not yet sufficient to support the concept that AhR-signaling has pathophysiological effects in human leishmaniasis. Extensive studies using different human monocyte/macrophage populations and parasite strains are needed to answer this question.

Thus, as *in vitro* data on AhR-signaling suggest both positive and negative effects on macrophage activation, and our own data suggested a positive regulation of *Leishmania*-induced cytokine secretion by AhR, we then investigated the relevance of AhR-signaling during *L. major* infection *in vivo*. Analyzing the effect of AhR activation on cytokine expression in infected skin, we found that AhR agonist indeed also induced expression of *Tnf in vivo*. We also observed AhR-dependent regulation of *Cxcl10* and *Cxcl2*. We have previously demonstrated that *Tnf*, *Cxcl10* and *Cxcl2* are all more prominently expressed in the skin of resistant animals (5) and could therefore contribute to resistance. Other, already confirmed early regulatory cytokines like Il4, Il6, Il12, and Il1a (3, 5, 47) were not affected by ITE treatment.

Thus, AhR activity contributes to the early skin cytokine milieu. Since we previously had demonstrated the relevance of the early cutaneous cytokine milieu for the subsequent development of resistance (5, 6), we speculated that early AhR-dependent signaling also influences the course of infection. Indeed, when we treated susceptible BALB/c mice with AhR agonist ITE at the first day of infection we observed a small, but significant reduction in footpad swelling during the first and third week of infection. No more differences in footpad swelling were observed after 4w, but we found more than 4-fold reduced numbers of parasites in skin dLN. We also found a significant∼50% reduction of *L. major*-specific IL4 secretion by restimulated dLN-cells in the early phase of Th differentiation. The relative reduction in IL4 secretion by ITE treatment was still observable after 4w, albeit not as pronounced. We observed no concomitant increase of IFNγ secretion. The reduced levels of IL-4 could enhance macrophage effector functions and explain the lower numbers of living parasites in draining lymph nodes. Thus, we show significant and matching effects on footpad swelling, parasite levels and the Th2 response during the first weeks of infection after a very short and local treatment with non-persistent AhR agonist only at the time of parasite inoculation. Therefore, our experiments indicate that AhR activation in skin of susceptible mice enhances early resistance against *L. major* by diminishing the Th2 response, but is not sufficient to reverse the genetic susceptibility toward the parasite.

Significant involvement of AhR-signaling in immunity (23, 33, 48) is established for infection with e.g., influenza and toxoplasma. There are also some data on the relevance of AhR for experimental leishmaniasis. *Ahr* knockout mice on a resistant background showed increased resistance compared to wildtype animals (24). On the other hand, oral treatment of infected mice with persistent AhR ligand 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) lead to a slower disease progression and lower parasite numbers not only in BALB mice, but also in Skid mice lacking T-cells, indicating that TCDD-induced changes in AhR activity in innate immune cells are relevant for AhR-mediated resistance (35).

While these studies demonstrate that AhR-signaling can influence experimental leishmaniasis, seemingly both positively and negatively, and in case of peritoneal knockout macrophages, at the same time, they focus neither on the function of AhR-signaling during the early crucial phase of infection nor on the relevance of AhR-signaling at the site of infection. The work by Elizondo et al. using *Ahr* knockout mice on a resistant background found that systemic absence of AhR caused a decrease in FoxP3+ regulatory T-cells in infected animals in the 4th and 6th week of infection and a concomitant increase in IL10 secretion by restimulated pLN cells after 6w, while Leishmania Ag specific secretion of IFNγ and IL4 were not affected in infected animals (24). Moreover, *Ahr* knockout mice had higher serum levels of TNF in the blood prior to and also during infection. The source of TNF production was not specifically determined. Since in our experiments AhR was only activated locally in the early phase of infection prior to the development of the Th-cell response, these results are not necessarily contradictory to our observed beneficial effect of early local AhR activation, especially because there is evidence for AhR activity-dependent induction of TNF secretion in macrophage like cells (45). The permanent absence of AhR-signaling in all immune cells including T-cells does not allow for analyzing time- or location-dependent effects. In those studies using animals treated systemically with permanent AhR ligand TCDD, AhR is permanently activated. Since effects of TCDD were also seen in SCID mice, this argues for T-cell independent effects of AhR-signaling in experimental leishmaniasis. However, significant differences in parasite numbers were only observed after 4 weeks

using high doses of TCDD in a low dose infection model and may therefore reflect the prolonged activation of AhR in this model which is in contrast to the transient activation by ITE in our experiments. Also, there is recent evidence from *Ahr* knockout rats suggesting that the classical AhR ligand TCDD has AhR independent effects on a number of immune cells like $CD11^+$ cells (49). Thus, the results from experiments using the TCDD are not in contrast to our results indicating an effect of early local AhR-signaling on Th-cell differentiation.

Our data newly establish that the local signaling by AhR increases resistance early during experimental leishmaniasis. It may exert these effects by influencing mechanisms of both innate and adaptive immunity. It has long been known, that early TNF production is required for resistance (38–41). Thus, enhanced expression of TNF by infected GMΦ might contribute to the increased early resistance in our *in vivo* experiments. TNF is necessary to facilitate the leishmanicidal properties of macrophages by inhibiting the IL4-induced production of Arg1 (50). Here, the lesional macrophage phenotype of TNF-deficient animals closely resembled that of susceptible BALB/c mice, so AhR activation might ameliorate the low leishmanicidal phenotype by boosting local TNF secretion. However, this effect can only be responsible for the very early difference in footpad swelling, as ITE is not a persistent AhR ligand and we have shown that TNF levels do not remain higher in ITE-treated animals during the height of infection.

With respect to adaptive immunity, we used ITE, a non-persistent AhR agonist which does not lead to TCDD-induced lymphocyte suppression and toxicity. We demonstrate significantly reduced *L. major*-specific secretion of IL4 by dLN-cells and therefore a diminished early Th2-response. We already demonstrated that differences in the early skin micromilieu correlate with differences in cytokine secretion from dendritic cells in skin draining lymph nodes (5). Thus, a similar effect of AhR-signaling on adaptive immunity via the altered skin cytokine milieu and dendritic cells is possible. The exact mechanisms of AhR-dependent effects on adaptive immunity have to be revealed in further studies.

In summary, our experiments demonstrate the relevance of AhR-signaling for cytokine expression in murine macrophages. We also demonstrate that AhR antagonist treatment reduced *L. major* induced *TNF* mRNA expression in human monocytes, indicating a possible role of AhR-signaling in the response of human monocytes to this parasite. *In vivo* we demonstrate the relevance of AhR-signaling in skin during *L. major* infection and provide further evidence for a relevance of the early skin micromilieu in instructing resistance to *L. major*.

Importantly, we showed that local treatment with a non-toxic, non-persistent, physiological AhR ligand had beneficial effects on experimental leishmaniasis in susceptible mice, opening possibilities of further studies on the effects of AhR-signaling in infectious diseases like leishmaniasis.

DATA AVAILABILITY STATEMENT

The array data generated for this manuscript was uploaded to the NCBI GEO repository under accession number GSE127541: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127541.

ETHICS STATEMENT

Human monocytes were isolated from fresh human blood leukocyte reduction chambers of platelet apheresis sets from healthy, voluntary whole blood donations after informed consent of the donors according to the regulations of the blood bank of the University Hospital Münster. Leukocyte reduction filters were anonymized prior to delivery from the blood bank in line with the ethics code provided by the scientific and ethics committee of the University of Münster.

AUTHOR CONTRIBUTIONS

N-AM, JR, CS, and JE contributed to the design and conception of the study. JE acquired and analysed array data. N-AM acquired and analysed all other data. N-AM wrote the first draft of the manuscript. N-AM, JE, and CS wrote sections of the manuscript. N-AM, JR, CS, and JE 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.2019.02442/full#supplementary-material

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REVIEW
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Understanding *P. falciparum* Asymptomatic Infections: A Proposition for a Transcriptomic Approach

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Malaria is still a significant public health burden in the tropics. Infection with malaria causing parasites results in a wide range of clinical disease presentations, from severe to uncomplicated or mild, and in the poorly understood asymptomatic infections. The complexity of asymptomatic infections is due to the intricate interplay between factors derived from the human host, parasite, and environment. Asymptomatic infections often go undetected and provide a silent natural reservoir that sustains malaria transmission. This creates a major obstacle for malaria control and elimination efforts. Numerous studies have tried to characterize asymptomatic infections, unanimously revealing that host immunity is the underlying factor in the maintenance of these infections and in the risk of developing febrile malaria infections. An in-depth understanding of how host immunity and parasite factors interact to cause malaria disease tolerance is thus required. This review primarily focuses on understanding anti-inflammatory and pro-inflammatory responses to asymptomatic infections in malaria endemic areas, to present the view that it is potentially the shift in host immunity toward an anti-inflammatory profile that maintains asymptomatic infections after multiple exposures to malaria. Conversely, symptomatic infections are skewed toward a pro-inflammatory immune profile. Moreover, we propose that these infections can be better interrogated using next generation sequencing technologies, in particular RNA sequencing (RNA-seq), to investigate the immune system using the transcriptome sampled during a clearly defined asymptomatic infection.

Keywords: asymptomatic infection, malaria, immunity, cytokines, RNA-seq

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INTRODUCTION

Malaria is a significant public health burden with an estimated 219 million new cases and 435,000 deaths reported in 2017 (1). Nearly half of the world's population is at risk of contracting malaria, with tropical and subtropical areas showing the highest prevalence (2). Malaria infection is caused by protozoan parasites of the genus *Plasmodium* that affect humans. *P. falciparum* is globally the most deadly and is the most prevalent parasite in Africa (3). *P. falciparum* malaria ranges from severe to uncomplicated or mild and to the poorly understood asymptomatic infections. Such diverse outcomes are due to the intricate interplay between factors derived from the human host, parasite, and environment (4). At the genomic level, differences in gene expression by the host during host-parasite interactions may account for the various clinical manifestations (5).

Specifically, gene pathways that regulate cytokine signaling and complement regulation as well as the production of immunoglobulins have been implicated (6). A strong pro-inflammatory response has been associated with an increased risk of febrile malaria, severe malaria anemia (7) or cerebral malaria (8), while a weak response has been associated with asymptomatic infection (9). Hence, the balance between pro-inflammatory and anti-inflammatory cytokine production appears to be important in influencing the outcome of malaria infections. Identification of markers that can diagnose the clinical manifestations of *P. falciparum* infections, in addition to symptoms, is important in predicting prognosis and directing treatment strategies.

Malaria infections are mainly characterized by a recurrent cycle of fever and chills. Other symptoms include vomiting, shivering, convulsions, and anemia caused by hemolysis (10). In some cases, these symptoms are not observed, and the infection is described as asymptomatic in individuals without a recent history of antimalarial treatment (11). Once an individual is infected with the parasite, immune factors are tasked with reducing parasite numbers, i.e., anti-parasite immunity, and preventing manifestation of clinical symptoms, anti-disease immunity. In asymptomatic individuals, immunity is skewed toward anti-disease rather than anti-parasite immunity. The mechanisms behind this phenomena are still unclear and more studies are required to understand how anti-disease immunity is induced and its potential for application in vaccine development (12).

DEFINING ASYMPTOMATIC *Plasmodium falciparum* INFECTIONS

The study of asymptomatic infections is still hampered by the lack of standard criteria for defining these infections (4, 11). This is due to the wide range of definitions that complicates the comparison of results across studies (**Table 1**). The most basic definition seems to be the presence of parasitemia and the absence of malaria symptoms, mainly fever (axillary temperature $< 37.5°C$) (14, 19, 20). This definition is ambiguous and most studies have modified it by incorporating strict inclusion criteria. Laishram et al. (4) summarized the diagnostic criteria used to define asymptomatic individuals in different studies and made several recommendations. They suggested the use of longitudinal follow ups, quantifying parasitemia rather than reporting its presence or absence and the use of PCR to identify asymptomatic infections in a population (4). Since then, the criteria have improved by incorporating the latest advancement in PCR, the loop-mediated isothermal amplification (LAMP), biomarkers to detect the parasite and use of cohorts that ensure reliable information about clinical history and follow ups. This allows for the exclusion of those who experienced symptoms in the recent past and then sought treatment. However, there is no consensus on the duration of history and it ranges from 2 weeks to 1 month (5, 14, 20). The longitudinal follow-ups after diagnosis reduces the chances of "false" asymptomatic parasitemia that are defined during *P. falciparum* incubation toward a clinical outcome. The duration of follow-up varies depending on whether the study is interested in asymptomatic parasitemia or the eventual symptomatic outcome (5, 14). The method for asymptomatic parasitemia diagnosis is also important. Microscopy, with a detection threshold of $\sim$50 parasites μl^{-1}, may miss subpatent infections, while others use PCR whose sensitivity can extend to below one parasite μl^{-1} (21, 22). Studies in Kenya, Uganda and Brazil have reported a significantly high prevalence of asymptomatic parasitemia, as much as 6–7 times higher, using PCR when compared to microscopy (13, 23, 24). PCR has also helped to identify individuals with low-density parasitemia in low-transmission settings that were previously missed by microscopy (25). Although the use of PCR is technical and expensive, making it unrealistic in most field studies, it is important in improving the accuracy of diagnosing asymptomatic parasitemia (26). Interestingly, LAMP has been shown to accurately detect sub-microscopic asymptomatic *Plasmodium* infections (27). LAMP is cheap and easy to implement in a field setting as it does not require a thermocycler machine like PCR. In addition, several biomarkers such as lactate dehydrogenase, hemozoin and, in particular, Histidine-Rich Protein 2 that is utilized in rapid diagnostic tests (RDTs), have been used to diagnose malaria (28, 29). Hemozoin is an important metabolite of hemoglobin digestion by the malaria parasite and is associated with pathogenesis as well as inducing immunity to malaria (30–32). A hemozoin sensing assay has recently been shown to be 20 times more sensitive than RDTs in diagnosing *Plasmodium* species (33). It could be applied as a point of care test and more importantly in screening populations for asymptomatic individuals with submicroscopic parasitemia (33). More efficient diagnostic techniques are needed to effectively detect asymptomatic infections in various settings to improve the quality and reliability of data used in studying asymptomatic infections. **Table 1** outlines the different criteria used in transcriptomics studies to define asymptomatic *P. falciparum* infections.

UNDERSTANDING THE RISK OF DEVELOPING FEBRILE INFECTIONS

Asymptomatic infections can act as precursors to malaria illness (34). Mass drug administration (MDA) has been suggested as an effective way of treating chronic asymptomatic infections (35, 36). However, this may interfere with the immunity maintained by these infections, thus increasing the risk of developing clinical malaria in asymptomatic individuals (37). A study in Mali treated chronic asymptomatic individuals at the end of the dry season, followed them up during the subsequent rainy season and reported that treatment of asymptomatic infections is unlikely to influence the subsequent risk of developing clinical malaria (17). Similar findings were also reported in Burkina Faso (38). A risk-benefit analysis is required to determine the tradeoffs to inform the public health impact of MDA on asymptomatic infections. The possibility of developing febrile malaria among asymptomatic carriers has been shown to vary due to transmission intensity and age (**Figure 1**) (20, 39). A study in Kenya compared the risk of developing febrile malaria among

TABLE 1 | Examples of inclusion criteria used to define asymptomatic individuals in transcriptomic studies.

Country, year	Criteria for identifying asymptomatic cases	Study subjects (sample size)	Follow-up protocol, duration	References
Cameroon, 2009	Positive thick blood smear and afebrile. No history of fever and antimalarial treatment in the previous 1 and 2 weeks, respectively, at the time of mass screening	Children <12 years (13)	No follow-up	(5)
Mali, 2011	PCR-detected *P. falciparum* and no fever. No history of antimalarial or immunosuppressive medication in the last 30 days and helminths	Individuals >13 (5)	Bi-weekly and weekly surveillance for *Plasmodium infection* and malaria episode, respectively	(14)
Gabon, 2005	Thin and thick blood smear and no clinical symptoms	Children 0.5–6 years (ND)	Follow up for 5 consecutive days	(6)
Uganda, 2007–2008	Blood smear and no fever	Children 4–5 years (15)	Follow up for 7 days	(16)
Mali, 2006	Not defined	5–13 years (17)	Healthy baseline before the malaria season, 7 or 14 days after treatment of their first malaria episode of the ensuing malaria season, and a subset of children followed up to the 6-month dry season	(18)

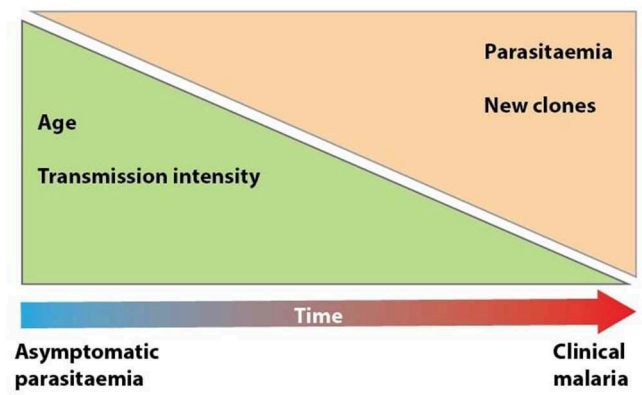

FIGURE 1 | Progression from asymptomatic to clinical malaria. Developing clinical malaria following an asymptomatic infection is influenced by host, parasite, and environmental factors. As individuals age and transmission intensity increases, the risk of developing clinical malaria decreases. This is primarily due to the development of acquired immunity in malaria endemic areas where exposure to repeated infection is common. An increase in parasitemia increases the risk of developing clinical malaria, while acquisition of new parasite clones increases the risk of developing symptoms due to lack of protective immunity against the new clones.

children (0–15 years) who were uninfected with *P. falciparum* and those with asymptomatic parasitemia. In lower transmission areas, asymptomatic parasitemia was linked to an increased risk of febrile malaria in children of all ages, while in moderate to high transmission areas, asymptomatic parasitemia was linked to a reduced risk of febrile malaria in children above 3 years (20). High asymptomatic parasitemia densities (≥2,500 parasites μL^{-1}) and every 10-fold increase in parasite density have been associated with an increased risk of developing febrile malaria, probably due to the underlying reduced host immunity (20, 39).

In addition, the transition from asymptomatic to symptomatic malaria may be influenced by the presence of new parasite genotypes to which an individual has not been previously exposed in the preceding asymptomatic infection, and hence they are likely to lack protective immune responses to the new genotype (40–42). *P. falciparum* infections in malaria endemic areas, primarily in sub-Saharan Africa, are characterized by the co-circulation of multiple *P. falciparum* clones in acute and persistent infections (43–47). This phenomenon is termed complexity of infection (COI). The presence of multiclonal infections has been associated with an increased range of anti-merozoite antibody responses. Together, COI and anti-merozoite antibody responses have been associated with a reduced risk of clinical malaria (48). Carriage of certain merozoite surface protein (*msp*) 1 genotypes have been associated with different clinical manifestations of malaria. In Nigerian children, *msp1* K1 and MAD20 alleles were associated with asymptomatic malaria and a minimal risk of becoming febrile (49). Similarly, using the *msp2* locus, the FC27 genotype was more prevalent in asymptomatic than in symptomatic Nigerian children (50). Other studies have shown that an increase in COI in children with asymptomatic infections is associated with an increased risk of febrile malaria in younger children, a lower risk in older children (51, 52) and in some cases, age had no influence (41, 53). Such conflicting findings on the role of COI in predicting febrile malaria could be due to methodology.

The traditional method for characterizing *P. falciparum* diversity uses nested-PCR with gel electrophoresis to detect polymorphisms in *msp1*, *msp2* and glutamine rich protein (GLuRP) (54) and recently by capillary electrophoresis (55). This technique has been shown to be limited in the number of variants detected within an individual infection (56, 57), is insensitive to less abundant variants and is not quantitative for relative proportions of circulating variants (57). Amplicon deep-sequencing overcomes these challenges as it is more sensitive

than capillary electrophoresis in detecting minority clones and is able to quantify individual *P. falciparum* clones (58). This technique also uses less DNA since adequate amounts of amplicons are obtainable directly from dried blood spots used for screening large sample sizes (59). With increased sensitivity and number of samples that can be genotyped, deep-sequencing promises to increase our understanding of the dynamics of *P. falciparum* COI and how this relates to the outcomes of febrile malaria. An understanding of how host immunity mediates the development of febrile malaria in asymptomatic carriers is required to better understand these infections.

IMMUNITY TO MALARIA

Individuals residing in malaria endemic zones often harbor asymptomatic infections and are clinically immune due to exposure to multiple genetically complex *P. falciparum* infections over time (60). Children who have experienced repeated malaria episodes have a modified immune system (26, 61, 62) that is for instance characterized by an increased production of immunoregulatory cytokine IL-10 and activation of neutrophils, B cells and $CD8^+$ T cells (63). The immune system is involved in controlling disease outcome as exhibited by the fact that the parasitemia tolerated in high transmission settings is higher than that causing fever in low transmission settings (64). The chances of an infection being asymptomatic increases with age as repeated exposure to malaria leads to the development of partial anti-disease immunity (65). Unfortunately, the immunity developed is not sterile, only suppressing but not eliminating the infection leading to disease tolerance and asymptomatic infection (66, 67). This immunity may also be lost due to a lack of continuous exposure to the parasite, resulting in elevated pro-inflammatory responses and subsequently a high risk of illness (68).

IMMUNOMODULATION IN ASYMPTOMATIC MALARIA

Modulation of immune responses has been associated with different clinical malaria manifestations (66, 69). The immune responses are mediated by cytokines that regulate inflammation and are thus involved in protective immunity. These cytokines include interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and IL-12 (70, 71). Overstimulation of the immune system leading to excessive production of these cytokines and activation of immune cells is detrimental to the host as they are likely to cause severe malaria symptoms through unknown mechanisms (72, 73). However, anti-inflammatory cytokines including IL-10, IL-27 and tissue growth factor beta (TGF-β) were shown to be involved in dampening pro-inflammatory cytokines, thereby minimizing disease severity (71, 74). The presence of anti-inflammatory cytokines, especially IL-10, suppresses parasite clearance, hindering the development of anti-parasite immunity, and clinical malaria (69), while promoting the development of asymptomatic infections (18). Elevated levels of IL-10 has also been linked to asymptomatic infections in pregnant women (75).

The production of anti-inflammatory cytokines has been shown to increase with repeated exposure to malaria, resulting in asymptomatic infection (18). Conversely, a lack of continuous exposure in historically exposed individuals can lead to the loss of anti-disease immunity (68, 76). This was exhibited by an increased production of pro-inflammatory cytokines and the proliferation of $CD4^+$ T cells (68). A study in Ugandan children revealed that the production of cytokines by $CD4^+$ T cells is influenced by prior exposure to malaria infections. $CD4^+$ T-cells in more exposed children were shown to produce higher levels of IL-10, while those in less exposed children produced higher levels of TNFα, hence promoting inflammation. The lack of TNFα production was associated with asymptomatic infections (9). A transcriptomic study in Mali described the activation of pro-inflammatory cytokine (IFN-γ, TNF, and IL-1β) production as being influenced by prior exposure to malaria, with asymptomatic infections having the least activation of these cytokines (14). Another study in Uganda revealed that frequent exposure to malaria infection causes decreased levels of pro-inflammatory cytokine (IFN-γ, TNF) producing $V\delta2^+$ γδ T cells and increased expression of immunoregulatory genes potentially dampening symptom development upon subsequent infections (16). In addition to exposure, age differences have also been suggested to modulate the immune system, with older children having lower anti-inflammatory and pro-inflammatory responses as compared to younger children (15). Lower levels of regulatory T cells (T regs) has also been observed in asymptomatic compared to symptomatic individuals (77, 78). Additionally, high levels of T regs have been associated with increased parasitemia, TGF-β production and the development of clinical symptoms (77, 79, 80). Lower T reg levels, on the other hand, may result in a decreased risk of developing symptoms, which translates to anti-disease immunity (78).

Unlike in asymptomatic infections, the Fulani ethnic group, who have reduced susceptibility to *P. falciparum* infection compared to sympatric tribes, have a higher ratio of pro-inflammatory to anti-inflammatory cytokines (81). The higher levels of pro-inflammatory cytokines have been implicated in causing the reduced symptomatic cases and parasite densities. In a transcriptomic study of their monocytes, increased upregulation of gene pathways involved in the production of pro-inflammatory cytokines in uninfected Fulani was observed, potentially priming the immune system to respond more effectively to *P. falciparum* infections (82). Thus, it appears that a balance between inflammatory and regulatory cytokines is important in achieving anti-disease immunity.

Antibodies also play a significant role in malaria protection. Seminal studies in monkeys and humans reported a reduction in fever and parasitemia following the passive transfer of serum or IgG antibodies from immune to non-immune subjects with acute malaria (83, 84). However, antibody responses to malaria seem to be short lived as exposure to malaria may not lead to the production of sufficient antigen-specific memory B cells (85). In contrast, a study on Swedish travelers previously treated for malaria maintained long-lasting memory B cells for 16 years without subsequent exposure (86).

TABLE 2 | A list of selected cytokines and immune cells showing their levels as reported in studies from Africa comparing malaria clinical outcome.

	Levels	Study site	Clinical comparison	References
IL-10	High	Uganda	Asymptomatic/symptomatic	(9)
	High	Ghana	Asymptomatic/uninfected	(75)
	High	Mali	Asymptomatic/febrile	(18)
	High	Gabon	Asymptomatic/mild	(90)
IFN-γ	Low	Uganda	Asymptomatic/febrile	(9)
	High	Gabon	Asymptomatic/mild	(90)
TNFα	Low	Uganda	Asymptomatic/febrile	(16)
	Low	Uganda	Asymptomatic/symptomatic	(9)
Tregs	Low	Uganda	Asymptomatic/febrile	(77)
	Low	Ghana	Asymptomatic/symptomatic	(78)
Vδ2$^+$ γδ T cells	Low	Uganda	Asymptomatic/febrile	(16)
Natural killer cells	Low	Kenya	Asymptomatic/uninfected	(26)

Higher titers of antigen-specific IgG have been observed in asymptomatic individuals compared to individuals with other malaria outcomes (87–89). Furthermore, high antigen-specific antibody responses were associated with high levels of IL-10 and IFN-γ in Gabonese children with asymptomatic *P. falciparum* infection, suggesting that the antibody response may exert protective immune mechanisms (90). Further studies of immune cells and cytokines (**Table 2**) are necessary to understand the mechanisms underlying immunomodulation and how this can be applied to confer malaria protection. It is evident that there is a complex interplay of various components of the immune system (**Figure 2**), and one way of potentially interrogating this complexity is through an "omics" approach.

To add to this already complex immune process, co-endemicity of *P. falciparum* and helminths is very common in the tropics resulting in increased chances of co-infection (91). Interactions between the two parasites alter immune responses, thus influencing susceptibility to clinical malaria (92, 93). There are conflicting reports on the outcome of these interactions as some studies have reported enhanced severity (94, 95), others reduced severity (96, 97) yet others have revealed no association between helminth co-infection and malaria outcome (98, 99). These observations may be due to differences in the co-infecting helminth species (100), the host's level of immunity to *P. falciparum* (101) and differences in study design (91). Various helminths elicit different immune responses that have an impact on the immunopathology of malaria as a result of the imbalance between pro-inflammatory and anti-inflammatory cytokines (102). However, there are contradictory reports on cytokine profiles resulting from helminths and *P. falciparum* co-infection. Co-infection with *Schistosoma haematobium* in Senegal was shown to offset this balance in an age-dependent manner in children and adults when compared to *P. falciparum* mono-infections. Co-infected children had higher levels of pro-inflammatory cytokines, IFN-γ and TNF-α, and were more likely develop febrile malaria, while co-infected adults had higher levels of similar pro-inflammatory cytokines as well as anti-inflammatory cytokines IL-10 and TGF-β and were more protected from malaria morbidity (103). In Uganda, IL-10 and IL-6 levels were elevated during *P. falciparum* and soil-borne helminth co-infection, while the level of TGF-β was reduced (104). The level of IL-10 was also elevated in asymptomatic *P. falciparum* Malian patients with filaria co-infection while IFN-γ, TNF-α, and IL-17 levels were reduced (105). There is growing evidence that *P. falciparum* and helminth co-infections have a profound effect on the host immune system, preventing the immune clearance of either parasite over the other. Although modulation of pro-inflammatory and anti-inflammatory cytokines responses has been suggested as a possible mechanism, more studies are needed to understand the roles of these cytokines and how other aspects of the immune system are involved.

ASYMPTOMATIC *P. falciparum* TRANSCRIPTOMICS STUDIES

A number of studies have examined the transcriptomics of asymptomatic *P. falciparum* infections and revealed interesting findings that add to the current phenotypic knowledge of these infections. The first study compared parasite gene expression patterns between 18 cerebral and 18 asymptomatic malaria infections in Cameroonian children using microarrays. The major difference was observed in genes coding for exported proteins, transcriptional factor proteins, proteins involved in protein transport, variant surface antigen (VSA) proteins such as *P. falciparum* erythrocyte membrane proteins (PfEMP) and repetitive interspersed family (RIFIN) (5). A more recent study in Gabon compared the host transcriptomic profiles between children with uncomplicated, asymptomatic, severe and cerebral malaria and identified 36 genes, among 4,643 transcripts, which are specifically regulated during asymptomatic infections. These genes are involved in nucleotide binding and RNA processing alluding to gene regulation via chromatin remodeling as a potential mode of maintaining asymptomatic infections (6). Chromatin remodeling changes the chromatin architecture, making condensed genomic DNA accessible to the transcription proteins and thereby regulating gene expression. This was shown to influence activation of immune cells such as monocytes and macrophages (106). It is hypothesized that during asymptomatic infections, chromatin remodeling decreases the expression of immunoglobulin genes leading to reduced antibody mediated responses (6). An opposite effect of chromatin remodeling is observed in the Fulani ethnic group. Chromatin remodeling may have resulted in stronger transcriptional activity in the monocytes leading to a pro-inflammatory state and reduced susceptibility to malaria infections when compared to sympatric ethnic groups (82). Pre- and post-infection profiles of Malian adults who were either febrile, asymptomatic or naïve were compared. Interestingly, asymptomatic individuals had the least transcriptional changes in gene pathways that are regulated by pro-inflammatory cytokines,

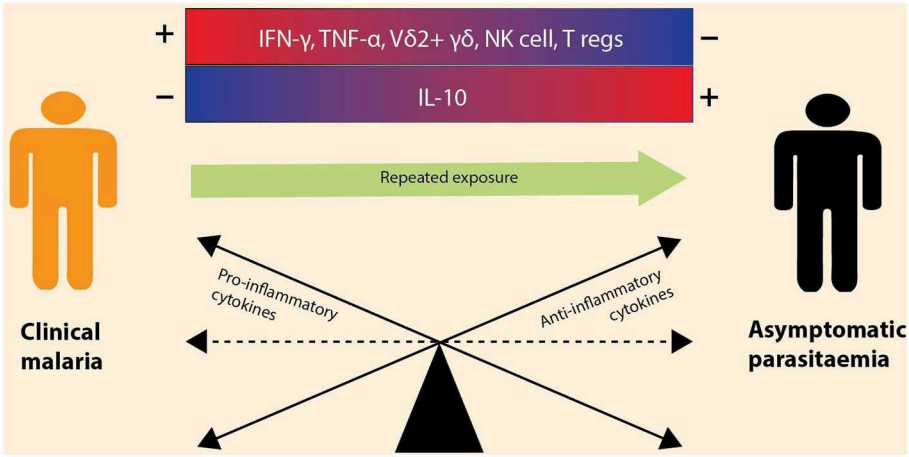

FIGURE 2 | Immune modulation during asymptomatic vs. symptomatic malaria infections. The outcome of a malaria infection is influenced by the balance between anti-inflammatory and pro-inflammatory cytokines. Clinical malaria is the result of elevated (+) production of pro-inflammatory cytokines (e.g., IFN-γ, TNF-α) and increased levels of immune cells (e.g., Vδ2$^+$ γδ, NK cells, and T regs) and the downregulation (–) of anti-inflammatory cytokines (e.g., IL-10). However, with repeated malaria exposure, the immune balance shifts toward an increased production of anti-inflammatory cytokines, leading to asymptomatic infection. The cytokines are encoded by immune genes, thus differential expression of these genes depicts that there is a balance between anti-inflammatory and pro-inflammatory cytokines.

IFN-γ, TNF, and IL-1β (14). This was probably caused by the downregulation of genes encoding pro-inflammatory cytokines, as observed in their lower levels in individuals with frequent exposure to *P. falciparum* in Mali (18). Chronic exposure to *P. falciparum* was shown to cause increased expression of immunoregulatory genes in Vδ2$^+$ T cells in Ugandan children (9). The results from the various transcriptomic studies in asymptomatic infections tend to agree that gene regulation mechanisms, either transcription factors or changes in the chromatin structure, may be involved in regulating inflammatory mechanisms that maintain malaria infections in the asymptomatic state. This can be confirmed by studying chromatin accessibility of gene regions that encode inflammatory regulators. Various techniques have been developed to assess genome-wide chromatin accessibility, with the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) proving to be the most effective (107). This technique has been applied in examining chromatin accessibility in *P. falciparum* and revealed insights into how the accessibility of specific transcriptome regulatory regions, i.e., promoter regions, are directly associated with an abundance of their corresponding transcripts (108, 109). The identification of regulatory regions that regulate expression of inflammatory genes in asymptomatic infections is important, as it would improve our understanding of how anti-disease immunity is maintained and reveal regions that can be targeted to achieve anti-parasite immunity and potentially, sterilizing immunity.

FUTURE PERSPECTIVES FOR ASYMPTOMATIC STUDIES

Asymptomatic malaria appears to be driven by host immunity, such that following multiple exposures to symptomatic infections an individual's immune system is potentially primed to control symptoms. Initially, at a young age, individuals in malaria endemic areas are likely to have high pro-inflammatory responses to control infection, which also results in the clinical manifestation of disease. With age, due to multiple exposure to infection, the immune system is perhaps trained and the balance shifts to a predominance of anti-inflammatory responses to control the infection with no clinical signs of disease, to a point that infection is tolerated and parasitemia is maintained. Since immunity to malaria is not sterile, there appears to be a trade-off between anti-parasite and anti-disease immunity, with the latter dominating in asymptomatic infections, where the focus is controlling disease rather than clearing parasites. This compromise in host immunity to control disease and not clear parasites is likely driven by the anti-inflammatory immune response. A transcriptomic approach to analyzing the cells and cytokines involved in the process would provide the necessary insight to unraveling the role of both anti- and pro-inflammatory responses to asymptomatic infections.

Though there is still a gap in our understanding of how the parasite remains in an asymptomatic state in individuals from malaria endemic areas, various studies have implicated genes responsible for gene regulation and chromatin remodeling and it is still unclear how host immunity is involved. High-throughput studies are needed to understand differential gene expression in immune cells that have shown differential activity in asymptomatic vs. symptomatic or uninfected states (5). Recently, single cell transcriptomics has emerged as a new approach in transcriptomic studies that characterizes individual cells, improving our ability to study cell to cell variability, unlike the conventional transcriptome method that assumes and treats cells from a certain tissue as homogenous (110). Reid et al. (111) used this technique to show transcription variation across all stages of the parasite life cycle and how genes involved in immune

evasion aid the parasite in transiting from the asexual stage in humans to its sexual stage in the mosquito (111). Properly designed asymptomatic *P. falciparum* single cell RNA-seq studies of individual immune cells and parasites with good criteria for defining asymptomatic infections hold the key to understanding these chronic and debilitating infections as well as host pathogen interactions in general.

Furthermore, there is a need for a consensus on how asymptomatic infections are defined. Longitudinal cohorts or follow ups over a period of 6 weeks, 4 weeks prior to the asymptomatic case and 2 weeks following the case, with no history or evidence of the individual having taken antimalarials and no evidence of fever 48 h before and after the case, are likely to be the most feasible approach to defining an asymptomatic infection. More sensitive tools for defining the presence of parasitemia to minimize missing sub-patent infections requires methods such as PCR, LAMP, or the newly described hemozoin sensing assay (33). With a clear definition of asymptomatic infections, further downstream analyses become possible. Exploiting next generation sequencing platforms to conduct amplicon deep sequencing and RNA-Seq has the potential to allow for a comprehensive analysis of asymptomatic infections to a scale not previously explored. The scale to which COI can be defined using amplicon deep sequencing improves the accuracy of determining COI in asymptomatic infections, the impact of COI on the risk of developing febrile disease and immunity. RNA-seq promises to unravel the complexities of the host immune response to malaria infection, describing at the transcriptomic level which molecules are likely to be involved in particular processes of controlling infection or, in the case of asymptomatic infections, maintaining parasitemia with no clinical symptoms. Of interest in this review was the role of anti- and pro-inflammatory cytokines in determining the course of infection. A focused analysis of transcripts related to these pathways would provide a better understanding of the role cytokines play in regulating the immune system and influencing malaria outcome. Notably, a better understanding of chromatin remodeling pathways is required to determine whether they are associated with particular gene transcripts, and ATAC-Seq will allow further interrogation of these pathways.

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KK and LO-O conceived, drafted, and reviewed the manuscript. KW drafted and reviewed the manuscript.

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SYSTEMATIC REVIEW
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Immune Responses to Gametocyte Antigens in a Malaria Endemic Population—The African *falciparum* Context: A Systematic Review and Meta-Analysis

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Background: Malaria elimination remains a priority research agenda with the need for interventions that reduce and/or block malaria transmission from humans to mosquitoes. Transmission-blocking vaccines (TBVs) are in development, most of which target the transmission stage (i.e., gametocyte) antigens Pfs230 and Pfs48/45. For these interventions to be implemented, there is a need to understand the naturally acquired immunity to gametocytes. Several studies have measured the prevalence of immune responses to Pfs230 and Pfs48/45 in populations in malaria-endemic areas.

Methods: We conducted a systematic review of studies carried out in African populations that measured the prevalence of immune responses to the gametocyte antigens Pfs230 and Pfs48/45. We assessed seroprevalence of antibody responses to the two antigens and investigated the effects of covariates such as age, transmission intensity/endemicity, season, and parasite prevalence on the prevalence of these antibody responses by meta-regression.

Results: We identified 12 studies covering 23 sites for inclusion in the analysis. We found that the range of reported seroprevalence to Pfs230 and Pfs48/45 varied widely across studies, from 0 to 64% for Pfs48/45 and from 6 to 72% for Pfs230. We also found a modest association between increased age and increased seroprevalence to Pfs230: adults were associated with higher seroprevalence estimates in comparison to children (β coefficient 0.21, 95% CI: 0.05–0.38, $p = 0.042$). Methodological factors were the most significant contributors to heterogeneity between studies which prevented calculation of pooled prevalence estimates.

Conclusions: Naturally acquired sexual stage immunity, as detected by antibodies to Pfs230 and Pfs48/45, was present in most studies analyzed. Significant between-study

heterogeneity was seen, and methodological factors were a major contributor to this, and prevented further analysis of epidemiological and biological factors. This demonstrates a need for standardized protocols for conducting and reporting seroepidemiological analyses.

Keywords: immunity, *Plasmodium falciparum*, gametocytes, Pfs230, Pfs48/45

INTRODUCTION

Progress has been made in controlling malaria with marked reductions in the global disease burden reported from 2000 onwards (1, 2). However, the 2018 World Malaria Report indicates stalling progress in malaria control in the past two years (1) which threatens the gains made in malaria control and hence efforts to develop new methods to control malaria must remain a priority. Vector control and treatment of acute malaria episodes remain key strategies. In addition to these malaria control strategies, there has been renewed interest in developing transmission-blocking vaccines (TBVs). While TBVs do not protect against clinical disease, they aim to reduce the infectiousness of mosquitoes thereby interrupting transmission (3, 4). The concept of a TBV was first demonstrated in early experiments in the late 1950s (5) and later in 1976 (6, 7) where reduced infectivity of gametocytes was demonstrated following immunization of avian hosts with inactivated gametocytes and gametes. Further experiments aimed to identify the targets of this transmission-reducing immunity by utilizing murine monoclonal antibodies to immunoprecipitate radioisotope-labeled female gametes (8, 9). The first TBV candidate antigens, Pfs25, Pfs230, and Pfs48/45 were thus identified, so named due to their observed molecular weight after separation by SDS-PAGE. Functional characterization of the Pfs230 and Pfs48/45 proteins has shown them to be essential for male gamete fertility and zygote formation (10, 11) while Pfs25 plays a role in ookinete to oocyst transition (9, 12).

Unlike Pfs25, which is expressed on the surface of activated female gametes and ookinetes post-fertilization in the mosquito midgut, Pfs230, and Pfs48/45 are expressed pre-fertilization in the mature gametocyte stages (13, 14) that are present in peripheral circulation awaiting uptake during a mosquito blood meal. The majority of these circulating gametocytes, however, are destroyed by the host immune system prior to transmission thus antigens present on the surface of the gametocytes are presented to the immune system (15, 16). Naturally acquired immune responses to Pfs230 and Pfs48/45 have been detected in the sera of individuals living in malaria-endemic areas (16–20) providing evidence that pre-fertilization sexual stage antigens are immune targets. Additionally, sera from individuals with high responses to Pfs230 and Pfs48/45 have been shown to exhibit transmission-reducing immunity (18, 20–23). Such naturally acquired transmission-reducing immunity (NA-TRI) has also been recently shown to reduce infectiousness in field settings (24). Though considered lead vaccine candidates, challenges in developing immunogenic vaccine constructs for Pfs230 and Pfs48/45 have delayed their evaluation in clinical trials. Both proteins contain a cysteine-rich domain whose disulphide-bonding pattern results in a complex tertiary structure that presents a challenge for producing properly folded recombinant protein (25, 26). The mapping of immunodominant epitopes (27, 28) and the development of various platforms for the production of properly folded Pfs230 and Pfs48/45 (29–32) have aided not only vaccine design but also seroepidemiological studies.

Several studies have now been carried out across multiple sites looking at naturally acquired immune responses to Pfs230 and Pfs48/45 (19, 20, 30, 33, 34) in a bid to improve our understanding of naturally acquired immunity (NAI) to sexual stage antigens. Classical indicators of parasite exposure such as age, transmission setting, malaria transmission season and parasite prevalence have been evaluated in a bid to describe the dynamics of sexual stage immunity. Discrepancies exist, however, in the observed associations of the aforementioned factors and seroprevalence of antibodies to sexual-stage antigens. For instance, there is no consensus on the impact of age on the development of sexual stage immunity, with some studies showing an increase in antibody prevalence with age (19, 33, 35) while other studies show no association with age (16, 36). Furthermore, while some studies have shown an increase in prevalence of responses in high transmission settings in comparison to low transmission settings (19, 34), other studies report higher responses in low transmission settings (36). Additionally, varied study designs and sampling protocols may affect the estimates of seroprevalence reported, making it difficult to provide an estimate of how common responses to sexual stage antigens are in the population.

Rationale

A better understanding of transmission-reducing immunity can offer important insights for the design, assessment and implementation of TBVs. This is of particular importance as though Pfs48/45 and Pfs230 are currently the most widely evaluated pre-fertilization TBV candidate antigens, evidence for the role of other antigens in the acquisition of NA-TRI (20, 36) would drastically increase the number of candidate antigens for evaluation. Understanding the dynamics of NA-TRI can provide important insights into the prioritization of candidate antigens for clinical testing. This work aims at providing a description of the prevalence of antibodies to sexual stage antigens and factors influencing sexual stage immune responses by analyzing responses to Pfs230 and Pfs48/45.

Objectives

Our study investigates naturally acquired transmission-reducing immunity in malaria-endemic populations in Africa. Specifically, it aims to:

a. Describe the prevalence of antibodies to the widely studied gametocyte antigens Pfs230 and Pfs48/45.
b. Identify factors associated with the acquisition of naturally acquired transmission-reducing immunity.
c. Describe the dynamics of naturally acquired transmission reducing immunity.

Research Question

This systematic review and meta-analysis aims to address the question "What is the seroprevalence and, at a population level, what are the factors that influence the development of naturally acquired anti-gametocyte immune responses in individuals living in *falciparum*-malaria endemic areas in Africa?"

METHODS

We performed a systematic review of studies of naturally acquired *Plasmodium falciparum* transmission-reducing immunity in Africa that reported the prevalence of antibodies to the widely studied gametocyte antigens Pfs230 and Pfs48/45. We followed the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines to conduct our analyses (37) and report our results according to the PRISMA (Preferred Reported Items for Systematic Reviews and Meta-Analyses) guidelines (38) (**Supplementary Table 1**). The study protocol is registered on PROSPERO (number CRD42019126701).

Study Design

We considered cross-sectional and longitudinal studies in our analyses. The inclusion of longitudinal studies that were spread over the malaria transmission season allowed for examination of transmission season as a potential modulator of sexual stage immune responses. We excluded hospital-based studies as they potentially would confound our results since these studies recruited participants with acute malaria infection. Our goal was to describe seroprevalence in a way that was generalizable at a population level.

Participants

The study population investigated was individuals living in malaria-endemic areas in Africa. We included studies recruiting both children and adults to be as representative as possible and our outcome was the development of antibodies to Pfs230 and/or Pfs48/45.

Search Strategy

The search strategy was based on the keywords: (pfs230 OR pfs48 OR pfs45) AND (antibodies OR immunity OR response) AND (plasmodium OR falciparum OR malaria). Reference lists of relevant studies were also searched for additional studies.

Data Sources, Studies Selection, and Data Extraction

Data Sources

Databases searched were MEDLINE/PubMed, SCOPUS, Web of Science, African Index Medicus, Embase, and African Journals Online from 1st February 2019 to 31st March 2019. We contacted study authors to provide prevalence data where it was not possible to extract the information directly from the published source. Alternatively, if raw data were available in public repositories, we used these data to estimate seroprevalence.

Study Selection

Criteria for study inclusion were: (1) studies reporting data from Africa (2) studies that measured antibody responses to Pfs230 and/or Pfs48/45. Studies from all years and written in all languages were included. Studies were excluded if: (1) they only reported antibody responses to non-*falciparum* antigens (2) they were vaccine, drug, or any other interventional trial (3) they analyzed responses in pregnant women (4) they did not measure antibody responses quantitatively (5) they sampled fewer than 30 participants (where studies recruited both children and adults, studies with fewer than 30 participants in each category were excluded). Where two studies had analyzed the same cohort, we considered the study where seroprevalence was evaluated in relation to a larger number of variables that were to be tested in the analyses.

Data Extraction

Data on seroprevalence to Pfs230 and/or Pfs48/45 were extracted from the studies using a standardized data extraction form. The data extraction form was developed to capture information on the study site, transmission intensity of the study site, season during which the participants were recruited, asexual and sexual parasite prevalence, study population, study design, age categories investigated, type of immunoassay used to detect immune responses, antigen coating concentration, serum dilution, source of antigen for the immunoassay, type of negative controls used, and method used to assess seropositivity.

Data Analysis

Heterogeneity between studies was assessed using the Cochran's Q, I^2, and H statistics with I^2 values of <30, 30–75, and $>75\%$ as cutoffs for low, moderate, and high estimates of heterogeneity, respectively. Sources of heterogeneity were explored through moderator analysis using sub-grouping and meta-regression. Conservative *p*-values were calculated using the "Knapp-Hartung" method. Additionally, we adjusted *p*-values for multiple comparisons using the Benjamini and Hochberg correction (39). We calculated the change in heterogeneity observed after each univariable analysis using the formula [(overall heterogeneity – residual heterogeneity/overall heterogeneity) * 100].

Variables explored in the meta-regression included: age group (broadly categorized as children [0–17 years of age] vs. adults [$\geq$18 years of age]), parasite prevalence, antigen source for immunoassay (recombinant protein vs. gametocyte extract), antigen coating concentration used in the immunoassay, and

seropositivity cut-off (2 or 3 standard deviations above the antibody reactivity of malaria naïve individuals or seronegative individuals identified using maximum likelihood methods to define Gaussian populations of low and high responders). Where a study reported seroprevalence for age-categories that slightly overlapped our pre-defined age categories (i.e., children 0–19 years of age or adults >16 years of age) and data were not available to reanalyze the age-category, we used the original study's defined age categories for children and adults in our analyses. For parasite prevalence, we used microscopy-based estimates as that was the parasite detection method used by majority of the studies.

To infer transmission intensity at the time of sampling in a uniform way across studies, we used data from the study by Snow et al. collected from across Africa from the early 1900s to 2015 that reported the predicted parasite rate standardized for 2–10-year olds ($Pf\mathrm{PR}_{2-10}$) (40). We used previously defined endemicity cut-offs (41) to categorize study sites as either hypoendemic; $Pf\mathrm{PR}_{2-10} \leq 10\%$, mesoendemic; $Pf\mathrm{PR}_{2-10} > 10$–50% or hyperendemic $Pf\mathrm{PR}_{2-10} > 50\%$. Information on the study site from where participants were sampled was then used to define the site's administrative region which was then used to guide the estimation of the $Pf\mathrm{PR}_{2-10}$ for that site.

For longitudinal studies, the seroprevalence estimates reported at the separate cross-sectional surveys over the follow-up period were combined to give an overall estimate. Where the data were seasonally spaced, each cross-section carried out at either the dry or the rainy season was considered separately in the univariable analysis. However, where a study measured responses at the peak and at the end of the rainy season, these data were pooled and analyzed as responses measured in the rainy season.

RESULTS

Flow Diagram of Studies Retrieved for the Review

Five hundred twenty-five potentially relevant studies were identified from the literature search, 205 of these were unique studies and these were then screened by title and abstract. From the screen, 34 studies were deemed to contain relevant information and after full-text evaluation and assessment against our inclusion criteria, 12 studies were included in the systematic review and meta-analysis. A summary of the selection process is provided in **Figure 1**.

Study Selection and Characteristics

The 12 studies were carried out across 17 study sites, majority of which were in West Africa (Burkina Faso, Senegal, Gabon, Cameroon, Ghana, and Mali) with only one study site in East Africa (Tanzania) and two study sites in Southern Africa (Zimbabwe) (**Table 1**). Ten articles (from 15 study sites) measured responses to Pfs230 and nine articles (13 study sites) measured responses to Pfs48/45. Six studies were longitudinal studies spread over the malaria transmission season with all but one measuring responses to both Pfs230 and Pfs48/45. Studies predominantly used ELISA as the immunoassay with only one study measuring responses using protein microarrays.

Pfs230

Seroprevalence

Ten studies from across 15 study sites in Africa analyzed immune responses to Pfs230. The range of seroprevalence estimates was quite wide, ranging from 6% reported by Stone et al. in Soumousso and Dande villages, Burkina Faso (20) to 72% reported by Amoah et al. (34) (**Figure 2**). Significant heterogeneity was observed between the studies ($I^2 = 97\%$; 95% CI: 96–98%; $p < 0.01$) therefore, a pooled prevalence estimate was not calculated.

Factors Associated With Reported Seroprevalence to Pfs230

We sought to explore how differences in the study population as well as in the immunoassay protocol employed affected reported seroprevalence to Pfs230 using meta-regression. Additionally, we tested whether the variables explored explained the between-study heterogeneity observed. The data presented in **Table 2** show the results of a univariable analysis. Compared to children, adults were associated with higher seroprevalence estimates (β coefficient 0.21, 95% CI: 0.05–0.38, $p = 0.042$). On the other hand, higher asexual parasite and gametocyte prevalence were associated with lower seroprevalence estimates, however, neither of these associations was statistically significant. Similarly, transmission intensity and sampling season (dry season vs. rainy season) were not significantly associated with seroprevalence estimates.

Of the methodological variables tested, in the six studies that used recombinant protein as the source of antigen (19, 20, 34, 35, 44, 45), studies using 1 μg/ml coating concentration of antigen in the immunoassay were associated with higher seroprevalence estimates (β coefficient 0.26, 95% CI: 0.09–0.43, $p = 0.042$). Moreover, compared to studies that used a seropositivity cut-off of 2 standard deviations (SD) above the mean response of seronegative individuals, studies that used a cut-off of 3 SD were associated with lower seroprevalence estimates (β coefficient −0.22, 95% CI: −0.37 to −0.06, $p = 0.042$). Though modestly significant ($p = 0.07$), antibody detection using the microarray platform was associated with higher seroprevalence estimates to Pfs230 (β coefficient 0.31, 95% CI 0.08, 0.55) in comparison to studies using indirect ELISA. Source of antigen (gametocyte extract vs. recombinant protein) was not significantly associated with seroprevalence estimates. Due to the limited number of studies included in the analysis and the fact that not all the variables were reported for each study, we did not attempt further multivariable meta-regression.

When we analyzed the degree of heterogeneity explained by the variables tested, none of the variables resulted in a decrease in residual heterogeneity below 90% precluding calculation of a pooled seroprevalence estimate. This was further confirmed by sub group analyses using age group, antigen coating concentration and seropositivity cut-off as variables (**Supplementary Figures 1–3**) where the heterogeneity

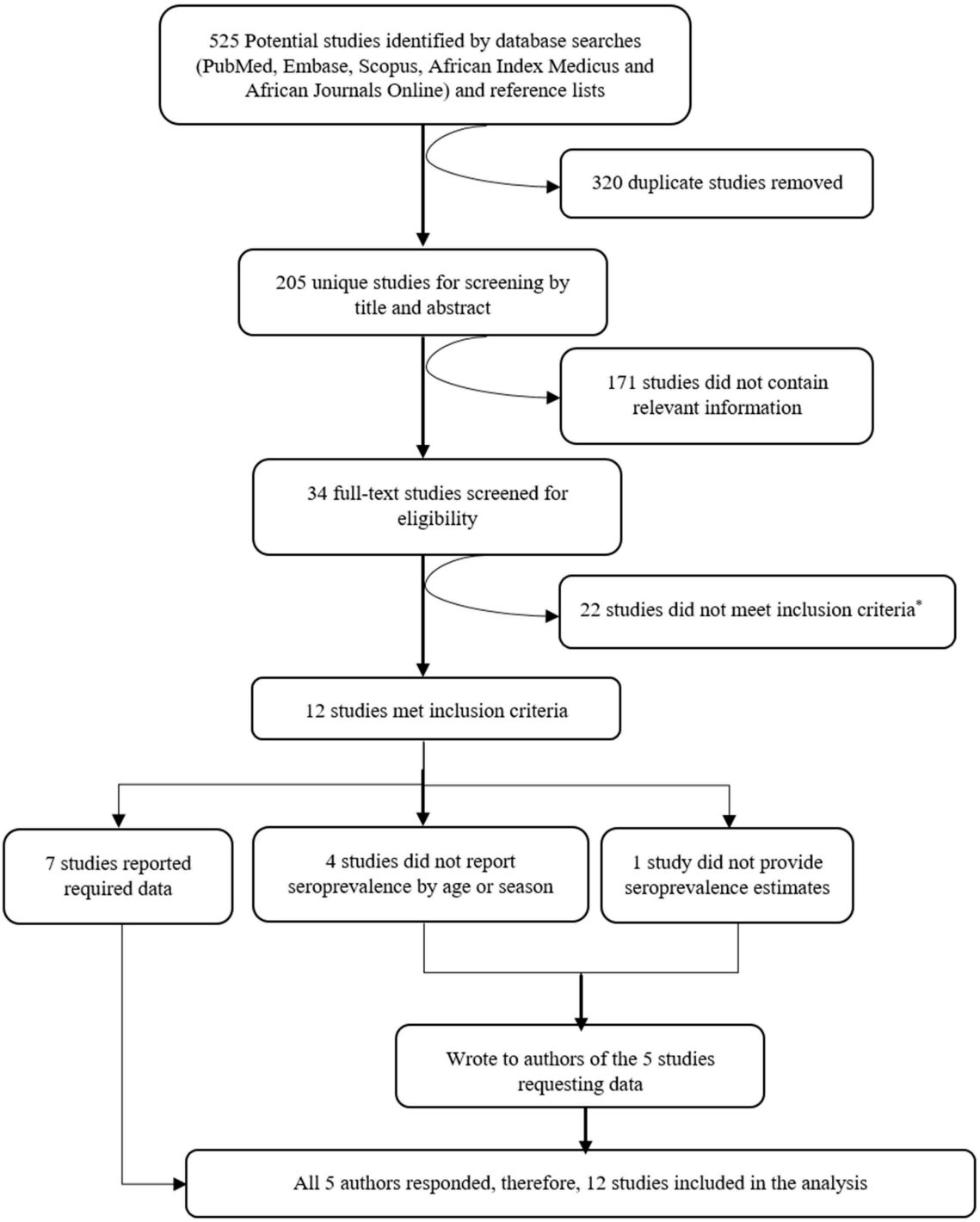

FIGURE 1 | Consort diagram showing the selection of studies to include in the systematic review and meta-analysis. Reasons for exclusion are included at each step. *Reasons for exclusion: six studies measured immune responses semi-quantitatively (four of these in the same population), 11 studies had a sample size of < 30, five studies were healthcare facility-based studies (i.e., primary care facilities or hospitals).

TABLE 1 | Characteristics of studies included in the systematic review and meta-analysis.

Study (Reference)	Year	Country	Region of study site[c]	Sample size	Age group (years)	Antigen detected	Seasonality tested (Y/N)	Assay	Seropositivity cut-off	Negative control[d]	Selective recruitment[e]
Amoah et al. (34)[a]	2018	Ghana (Abura)	Central	65	6–12	Pfs230	No	ELISA^{R}	2 SD	Naïve	No
Amoah et al. (34)[a]	2018	Ghana (Obom)	Greater Accra	75	6–12	Pfs230	No	ELISA^{R}	2 SD	Naïve	No
Lamptey et al. (35)	2018	Ghana	Greater Accra	338	2–65	Pfs230	Yes	ELISA^{R}	3 SD	Test sample	No
Stone et al. (20)[b*]	2018a	Burkina Faso	Hauts-Bassins	33	5–14	Pfs230 and Pfs48/45	No	ELISA^{R}	3 SD	Test sample	Yes
Stone et al. (20)[b*]	2018b	Burkina Faso	Centre-Nord	38	2–10	Pfs230 and Pfs48/45	No	ELISA	3 SD	Test sample	Yes
Stone et al. (20)[b*]	2018	Cameroon	Centre	140	5–16	Pfs230 and Pfs48/45	No	ELISA^{R}	3 SD	Test sample	Yes
Bansal et al. (42)	2017	Zimbabwe	Mashonaland Central	181	6–14	Pfs48/45	No	ELISA^{R}	2 SD	Naïve	No
Paul et al. (43)	2016	Zimbabwe	Manicaland	150	6–16	Pfs48/45	No	ELISA^{R}	2 SD	Naïve	No
Ateba-Ngoa et al. (44)[b]	2016	Gabon	Moyen - Ogooue	286	3–50	Pfs230 and Pfs48/45	No	ELISA^{R}	3 SD	Test sample	No
Jones et al. (19)[b]	2015	Burkina Faso	Nord	200	5–16	Pfs230 and Pfs48/45	Yes	ELISA^{R}	3 SD	Test sample	No
Jones et al. (19)[b]	2015	Ghana	Greater Accra	108	5–17	Pfs230 and Pfs48/45	Yes	ELISA^{R}	3 SD	Test sample	No
Jones et al. (19)[b]	2015	Tanzania	Tanga Region	202	3–15	Pfs230 and Pfs48/45	Yes	ELISA^{R}	3 SD	Test sample	No
Skinner et al. (33)[b]	2015	Mali	Koulikoro 3 and Bamako	225	2–25	Pfs230 and Pfs48/45	Yes	Microarray^{R}	2 SD	No Template	No
Miura et al. (45)	2013	Mali	Kayes 2	45	18–60	Pfs230	No	ELISA^{R}	3 SD	Naïve	No
Ouedraogo et al. (24)[b*]	2018	Burkina Faso	Centre-Nord	128	1–55	Pfs230 and Pfs48/45	Yes	Two-site ELISA^{Ge}	3 SD	Naïve	No
Ouedraogo et al. (16)[a]	2011	Burkina Faso	Centre-Nord	296	1–>20	Pfs230 and Pfs48/45	Yes	Two-site ELISA^{Ge}	2 SD	Naïve	No
Van der Kolk et al. (46)	2006	Cameroon	Centre	236	5–14	Pfs230 and Pfs48/45	No	Two-site ELISA^{Ge}	2 SD	Naïve	No

[a] *Seroprevalence data provided by authors upon request.*

[b] *Seroprevalence data calculated from data provided by original authors, or from data available on public repositories.*

[b*] *Citation also includes citation of repository from which data was retrieved.*

[c] *Administrative region of study site from which participants were drawn, this was used infer predicted parasite prevalence rates standardized in 2 – 10-year olds ($PfPR_{2-10}$) that was then used to assign transmission intensity at the time of sampling.*

[d] *Negative control refers to the comparator used to assign seropositivity in the immunoassay. Naïve – malaria naïve volunteers; Sample – a proportion of statistically – defined seronegative individuals; No template - a 'no DNA control' used to detect reactivity to the expression vector used to produce protein for the array.*

[e] *Selective recruitment refers to studies that only recruited parasite positive individuals for antibody measurements.*

[R] *Recombinant protein;* [Ge] *gametocyte extract.*

SD, standard deviation.

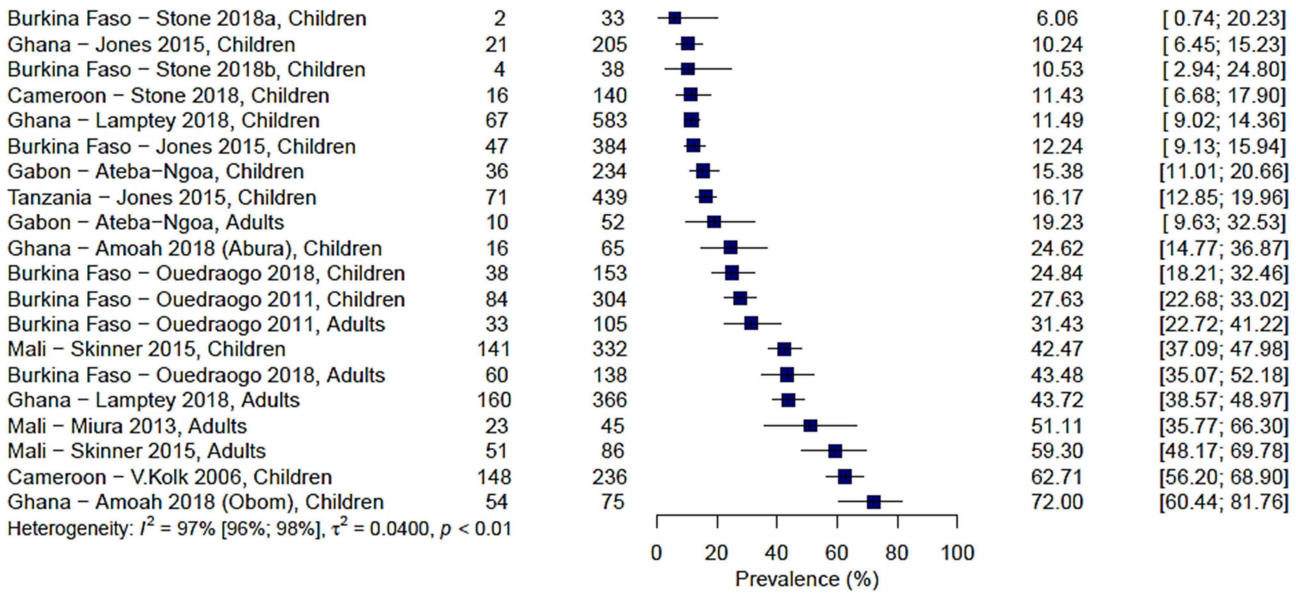

FIGURE 2 | Forest plot of the prevalence of antibodies to Pfs230 in endemic sera from Africa. Seropositive individuals were defined as study participants with an antibody reactivity above a set cut-off defined from seronegative individuals as measured in an immunoassay.

between studies in each category was still greater than our threshold of 75%.

Pfs48/45

Seroprevalence

A total of 9 studies carried out over 13 study sites measured immune responses to Pfs48/45. The range of seroprevalence estimates reported was 0% from Stone et al.'s study sites in Burkina Faso (20) to 64% reported by Paul et al. from their study in the Makoni district in Zimbabwe (43). As with Pfs230, there was significant heterogeneity between the studies, I^2 =96% (95% CI: 95–97%), and hence no pooled estimate was calculated (**Figure 3**).

We also carried out a pairwise comparison of seroprevalence estimates between Pfs230 and Pfs48/45 in five studies where both antigens were tested using the same protocol per study. Some studies reported higher responses to Pfs230 and others to Pfs48/45, and therefore we found no consistent pattern to suggest higher seroprevalence estimates to either antigen (**Supplementary Figure 4**).

Factors Associated With Reported Seroprevalence to Pfs48/45

As with Pfs230, we assessed how differences in the study population and in the immunoassay protocol affect reported seroprevalence to Pfs48/45 using meta-regression. From the univariable analysis, adults were associated with modestly higher seroprevalence estimates (β coefficient 0.07, 95% CI: −0.12–0.27) as with Pfs230, however, this was not statistically significant (p = 0.49). Higher parasite prevalence was associated with lower seroprevalence estimates, and this was statistically significant for gametocyte prevalence (β coefficient −0.003, 95% CI: −0.005–0.002, p = 0.003). Similar to Pfs230, transmission intensity and sampling season were not significantly associated with seroprevalence estimates.

Type of immunoassay was significantly associated with seroprevalence estimates as higher seroprevalence was reported where microarray was used in comparison to indirect ELISA (β coefficient 0.36, 95% CI: 0.15–0.56, p = 0.016). Furthermore, studies using 1 μg/ml coating concentration of antigen in the immunoassay were associated with higher seroprevalence estimates (β coefficient 0.30, 95% CI: 0.06–0.54, p = 0.043) while studies that used a seropositivity cut-off of 3 SD were associated with lower seroprevalence estimates (β coefficient −0.26, 95% CI: −0.39, −0.12, p = 0.003). We did not have statistical power to carry out multivariable meta-regression.

Gametocyte prevalence explained a high degree of heterogeneity (i.e., 25%), resulting in a residual heterogeneity of 71%, suggesting that seroprevalence was lower at higher gametocyte prevalence. We then decided to carry out a sub-group analysis of the four studies reporting gametocyte prevalence data (16, 19, 20, 24) with grouping by gametocyte prevalence coded as a categorical variable: <10, 10–50%, and >50% (**Figure 4**). We found that the observed association of decreased seroprevalence with increased gametocyte prevalence was highly influenced by Stone et al.'s study, where 99% of children sampled were gametocyte positive but reported low seroprevalence to Pfs48/45. This was confirmed by sensitivity analysis carried out in the absence of the Stone et al. study (**Supplementary Table 2**). Therefore, we do not have strong evidence of an association between seroprevalence and gametocyte prevalence when

TABLE 2 | Univariable meta-regression analysis of factors influencing reported seroprevalence to Pfs230.

	No. of studies (No. of Sites)	Coefficient (β)	Lower CI	Upper CI	*p*-value*	Residual I^2	I^2 change (%)
Age							
Children (ref.)	10 (14)						
Adults	6 (6)	0.21	0.05	0.38	**0.04**	95.36	**2.09**
Asexual parasite prevalence	6 (10)	−0.001	−0.005	0.002	0.51	95.37	2.08
Gametocyte prevalence	4 (8)	−0.002	−0.004	0.001	0.38	92.54	4.50
Transmission intensity							
Mesoendemic (ref.)	7 (8)						
Hyperendemic	6 (7)	−0.06	−0.23	0.11	0.51	96.18	1.25
Season							
Dry (ref.)	6 (9)						
Rainy	5 (7)	0.07	−0.12	0.27	0.51	96.24	1.19
Assay							
ELISA (ref.)	6 (11)						
Microarray	1 (1)	0.31	0.08	0.55	0.07	95.29	2.17
Two-site ELISA	3 (3)	0.12	−0.06	0.29			
Antigen							
Gametocyte extract (ref.)	3 (3)						
Recombinant protein	7 (12)	−0.06	−0.25	0.13	0.51	96.31	1.12
Antigen concentration$^+$							
0.1 μg/ml (ref.)	3 (7)						
1 μg/ml	3 (4)	0.26	0.09	0.43	**0.04**	93.52	**3.98**
Seropositivity cut-off							
2 SD (ref.)	4 (5)						
3 SD	6 (10)	−0.22	−0.37	−0.06	**0.04**	95.16	**2.30**

**p-values adjusted using the Benjamini and Hochberg correction for multiple testing; values in bold $p < 0.05$.*
$^+$ Antigen concentration was only tested for studies using recombinant protein as antigen source.
CI, confidence interval; SD, standard deviation.

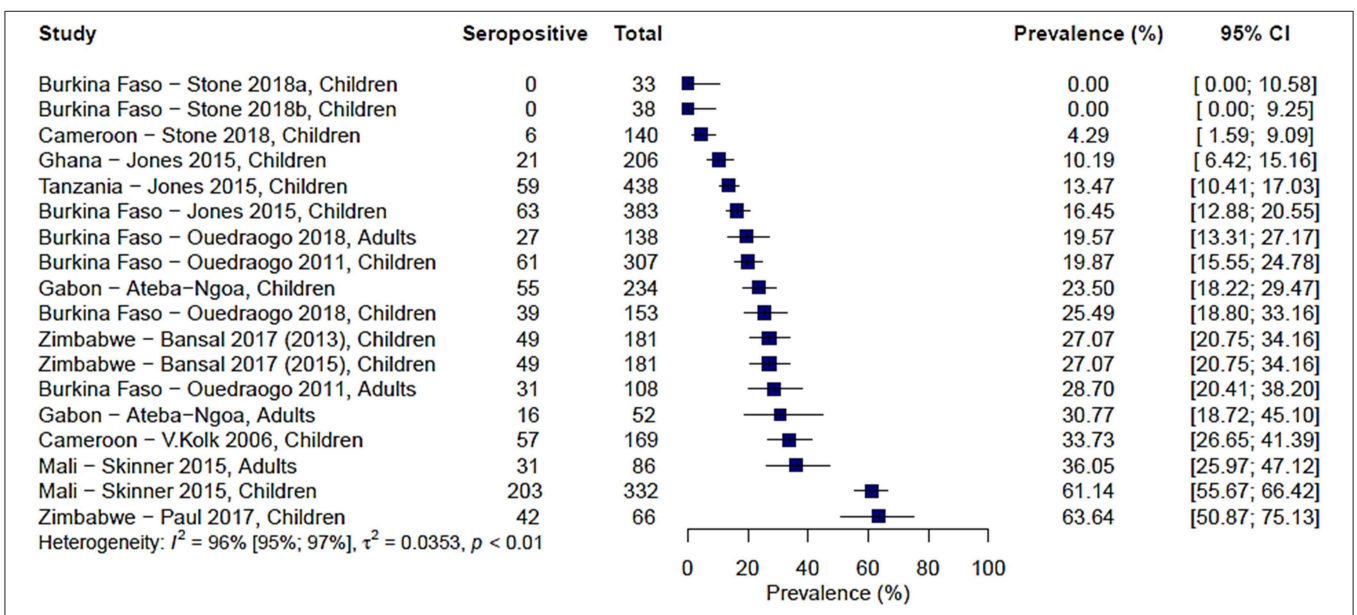

FIGURE 3 | Forest plot of the prevalence of antibodies to Pfs48/45 in endemic sera from Africa. Seropositive individuals were defined as study participants with an antibody reactivity above a set cut-off defined from seronegative individuals as measured in an immunoassay.

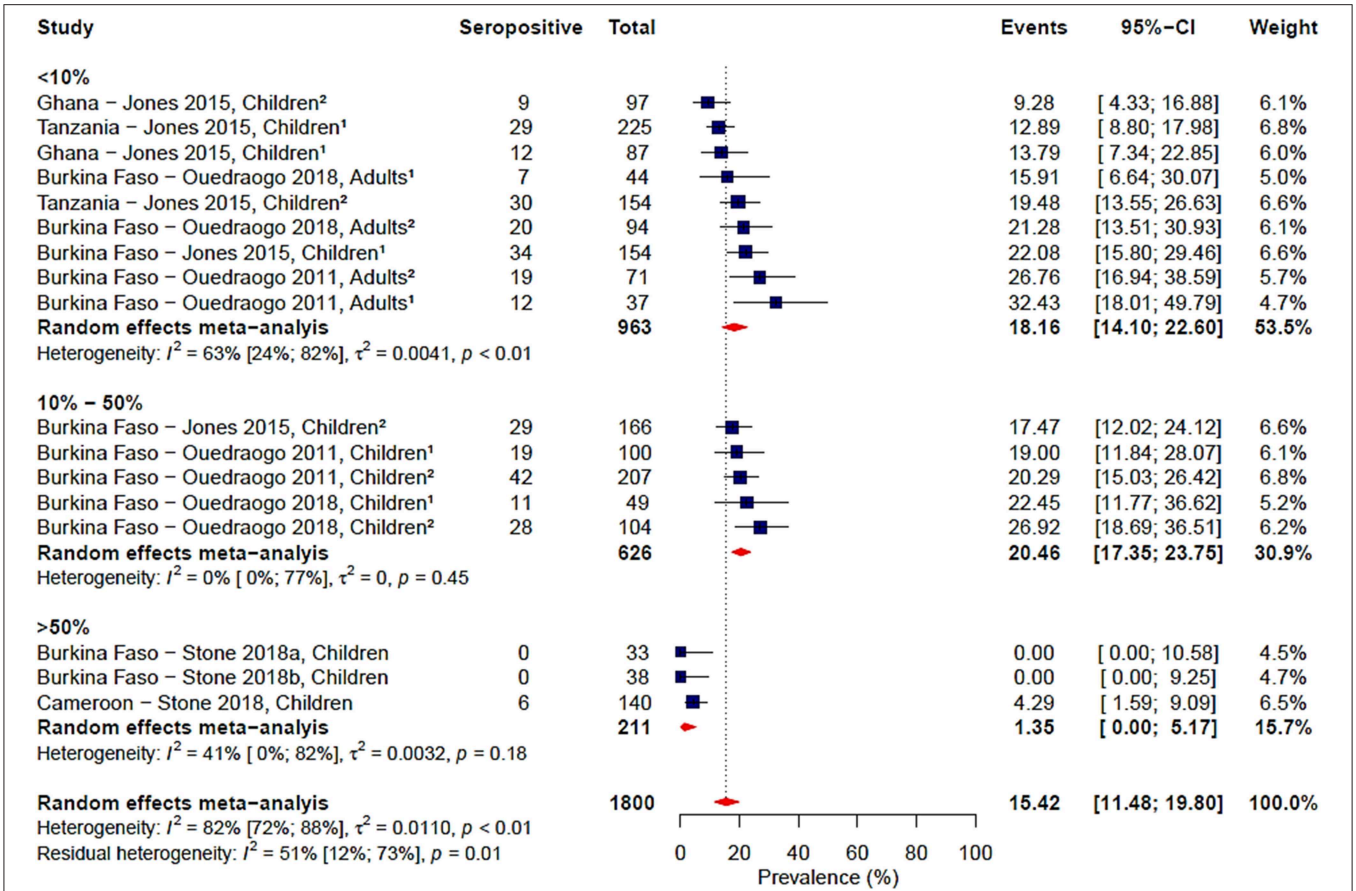

FIGURE 4 | Forest plot of the prevalence of antibodies to Pfs48/45 in endemic sera from Africa grouped by gametocyte prevalence. Seropositive individuals were defined as study participants with an antibody reactivity above a set cut-off defined from seronegative individuals as measured in an immunoassay. [1]Participants samples during the dry season; [2]Participants sampled during the rainy season.

comparing these studies. Although assay, antigen coating concentration and seropositivity were statistically significant predictors in the meta-regression, sub-group analysis by assay, antigen coating concentration or seropositivity cut-off did not reduce the heterogeneity to below 75% as seen in **Table 3** and **Supplementary Figures 5–7**.

Risk of Bias

As the studies included in these analyses were observational and non-comparative, we did not feel it appropriate to test for publication bias. We observed that studies reporting low prevalence were just as likely to report their results as studies reporting a high prevalence of antibodies to Pfs230 and Pfs48/45 as neither of these outcomes are linked to a statistically significant result that would be considered desirable or undesirable.

DISCUSSION

With a renewed interest in developing a malaria TBV, a better understanding of naturally acquired immunity (NAI) to malaria is required to aid vaccine design, evaluation and implementation. We carried out a systematic review and meta-analysis of studies that evaluated naturally acquired immune responses to the widely study vaccine candidate antigens Pfs230 and Pfs48/45 in African populations. By combining results from different studies carried out across the continent, we aimed to define the prevalence of NAI to the two well-characterized sexual stage antigens. Our analysis largely focused on age, transmission intensity, season and parasite prevalence as markers of malaria exposure, and sought to describe their association with seroprevalence to Pfs230 and Pfs48/45 at the population level.

When all studies were examined in combination, the reported seroprevalence to both antigens ranged from 0% (20) to 64% (45) for Pfs48/45 and from 6% (19) to 72% for Pfs230 (34). Between-study heterogeneity did not allow for the pooling of studies to arrive at a single reliable seroprevalence estimate for either antigen. We therefore sought to explore potential factors affecting seroprevalence and their possible contribution to the heterogeneity observed. Factors we considered were age, transmission setting, sampling season and parasite prevalence. Furthermore, noting that there was considerable variation in study design, we also considered methodological factors in the analysis.

TABLE 3 | Univariable meta-regression analysis of factors influencing reported seroprevalence to Pfs48/45.

	No. of Studies (No. of Sites)	Coefficient (β)	Lower CI	Upper CI	*p*-value	Residual I^2	I^2 change (%)
Age							
Children (ref.)	9 (13)						
Adults	4 (4)	0.07	−0.12	0.27	0.49	94.90	−0.18
Asexual parasite prevalence	4 (8)	−0.003	−0.006	0.0003	0.11	91.41	3.96
Gametocyte prevalence	4 (8)	−0.003	−0.005	−0.002	**0.003**	70.82	**25.24**
Transmission intensity							
Hypoendemic (ref.)	1(1)						
Mesoendemic	5 (6)	−0.47	−0.89	−0.06	0.11	93.91	0.87
Hyperendemic	5 (6)	−0.38	−0.80	0.04			
Season							
Dry (ref.)	4 (6)						
Rainy	6 (8)	0.07	−0.09	0.24	0.47	93.12	1.70
Assay							
ELISA (ref.)	5 (9)						
Microarray	1 (1)	0.36	0.15	0.56	**0.016**	91.99	**2.89**
Two-site ELISA	3 (3)	0.09	−0.07	0.24			
Antigen							
Gametocyte extract (ref.)	3 (3)						
Recombinant protein	6 (10)	−0.01	−0.19	0.17	0.91	94.91	−0.19
Antigen concentration$^+$							
0.1 μg/ml (ref.)	3 (7)						
1 μg/ml	2 (2)	0.30	0.06	0.54	**0.043**	92.65	**2.20**
Seropositivity cut-off							
2 SD (ref.)	5 (5)						
3 SD	4 (8)	−0.26	−0.39	−0.12	**0.003**	91.38	**3.54**

**p-values adjusted using the Benjamini and Hochberg correction for multiple testing; values in bold $p < 0.05$.*
$^+$Antigen concentration was only tested for studies using recombinant protein as antigen source.
CI, confidence interval; SD, standard deviation.

The influence of age on NAI to sexual stage antigens has been the subject of much debate with some studies showing no age-dependent acquisition of immune responses (16, 36, 47) while others demonstrate increasing antibody prevalence with age. NAI to asexual stage antigens, for instance, merozoite surface proteins (48, 49) or the infected erythrocyte protein family *Plasmodium falciparum* erythrocyte membrane protein 1 (50), has been more extensively studied than immunity to sexual stage antigens and reports indicate that immune responses increase with age. This may reflect the time taken to acquire a repertoire of antibodies, particularly to clonally variant antigens such as PfEMP1, and/or the gradual acquisition of long-lived plasma cells and memory B cells with repeated parasite exposure (49, 51). This meta-analysis found a modest association between increased age and an increase in the prevalence of antibodies to the gametocyte antigens Pfs230 and Pfs48/45 which was statistically significant for Pfs230 (**Table 2**). Some studies have argued that immune responses to sexual stage antibodies are short-lived [potentially due to a largely T-cell independent response (52)] and reflect recent rather than cumulative exposure to gametocytes (16, 36). Though not conclusive, this analysis suggests that there may be a case for long-lived responses to sexual stage antigens. Indeed, Ouedraogo et al. in their recent study found higher prevalence and density of antibodies to Pfs230 and Pfs48/45 in adults which positively correlated with higher transmission-reducing activity (24).

Our combined analysis did not find a definitive association between sampling season and seroprevalence to Pfs230 or Pfs48/45, though there was a trend toward higher seroprevalence during the rainy season. In the individual studies considered in the analysis (16, 19, 24) boosting of responses to gametocyte antigens following the malaria transmission season associated with increased seroprevalence in responses to both antigens. Boosting of responses to pre-fertilization sexual stage antigens during natural malaria infection is an argument put forth in favor of prioritizing antigens such as Pfs230 and Pfs48/45 for TBV design (29, 30, 33). Maintaining high titers of transmission-reducing responses would be essential in reducing malaria transmission potential. The study by Ouedraogo et al. (24) that directly assessed the impact of NAI to Pfs230 and Pfs48/45 on infectiousness to mosquitoes found decreased infectiousness to mosquitoes during the malaria transmission season that also

coincided with boosted responses to the two antigens. This observation may provide evidence to support natural boosting of TBV responses.

In addition to looking at age and season, we also evaluated the relationship between transmission intensity and seroprevalence. Though seroprevalence estimates in individual studies conducted across multiple study sites described an increase in seroprevalence with increased transmission settings, we were not able to demonstrate this in our meta-analysis. Likewise, we did not observe an association between asexual parasite or gametocyte prevalence and seroprevalence to either Pfs230 or Pfs48/45.

Substantial heterogeneity between studies persisted even after sub-group analysis by our pre-specified variables relating to malaria exposure, and so we also investigated the contribution of methodological variability to the observed heterogeneity. Studies differed in their source of antigen, choice of immunoassay, assay protocol and seropositivity cut-off determination. Early studies of NAI to Pfs230 and Pfs48/45 relied on whole antigen for the determination of immune responses owing to the difficulties in producing recombinant protein for analysis. Assays with whole antigen involve a two-site ELISA where an epitope-targeted monoclonal antibody is used to "capture" the target antigen for detection by antibodies present in the immune sera. Such assays, however, are reportedly less sensitive (19, 30) which could lower seroprevalence estimates. In our analysis, however, we did not observe an influence of antigen source on seroprevalence. We did, however, find that type of immunoassay influenced seroprevalence with seroprevalence measured using protein microarray reporting higher results than either indirect ELISA or two-site ELISA. This is likely due to the greater dynamic range afforded by microarray (53) that may allow better distinction between seropositive and seronegative individuals. We believe that the field will gradually move to high-throughput multiplex methods such as microarray or Luminex, although there will remain a place for ELISAs when large populations are examined for single-antigen responses.

We found that studies using a lower antigen coating concentration reported lower seroprevalence estimates. Seroprevalence to sexual stage antigens is lower than to asexual stage antigens (23, 54), possibly due to lower immunogenicity of these antigens or a higher ratio of circulating asexual parasites to gametocytes (33). For this reason, lower antigen coating concentration may reduce assay sensitivity, thus it is paramount that studies optimize antigen coating concentration and serum dilution combinations prior to measuring immune responses. Subsequent to measuring immune responses, studies need to clearly define seronegative and seropositive participants to estimate seroprevalence. Typically, this is done by defining cut-offs based on either two or three standard deviations (SD) of the mean antibody responses in malaria naïve individuals or in a statistically-defined population of low responders. We found that studies using a 3 SD cut-off reported lower seroprevalence, potentially a reflection of the higher stringency in comparison to a 2 SD cut-off.

What recommendations could be made? First, seropositive status should be assigned by comparison with a negative control group (i.e., a population with no malaria exposure). In practice, the most widely accessible means of doing this is using malaria naïve European or American sera as a reference. Second, given the variability of assays, we would recommend the use of more stringent cut-offs and therefore proposed the mean plus 3 SD of a malaria-naïve population to be used to determine seropositivity. Establishing a recognized and broadly accepted "gold-standard" for seropositivity estimates would allow a more robust comparison between seroepidemiological studies (55). The increased availability of recombinant protein with defined conformational properties for use in immunoassays makes the goal of standardized methodologies more attainable.

Heterogeneity was more evident in the studies reporting immune responses to Pfs230 than to studies analyzing Pfs48/45. We hesitate to ascribe this to biological differences between the two antigens, but rather to persisting heterogeneity in the studies that analyzed immune responses to Pfs230. Factors associated with seroprevalence estimates to the two antigens were largely methodological rather than epidemiological underscoring the importance of more standardized methods for seroepidemiological studies of sexual stage antigens. With the increasing number of assays performed for seroepidemiology studies, including but not limited to ELISA, protein microarrays, Luminex and AlphaScreen, there is need for minimum reporting parameters to ensure reproducibility, standardization, and to an extent generalizability of findings. For the analysis of Malaria Immunoepidemiology Observational Studies (MIOS), Fowkes et al. (56) have provided reporting standards. In addition to these standards, we propose that the following minimum methodological criteria be adopted in lieu of a gold standard: (1) use of recombinant proteins (with indications of the protein expression system utilized, protein region targeted—full length vs. fragments) and if gametocyte/gamete extract is used, a further analysis of responses to a dominant recombinant antigen (e.g., Pfs230 and/or Pfs48/45); (2) use of a 3 SD cut-off to assign seropositivity from a population of naïve controls; and (3) mention of antigen coating concentration and serum dilutions used.

Summary of Main Findings

In summary, from the studies analyzed, this systematic review shows that the range of reported seroprevalence to Pfs230 and Pfs48/45 varies widely across populations. Of the factors thought to influence seroprevalence, we found a case for age as an important determinant of seroprevalence. This is particularly important as the demonstration that functional TRI likely involves antigens other than Pfs230 and Pfs48/45 (18, 20, 21, 46), and with the identification of new TBV candidate antigens (20), criteria for identifying and prioritizing candidate antigens would be required. Screening for NAI and selecting antigens which show increased recognition with age could eliminate antigens that are simply markers of gametocyte exposure, thus prioritizing more important candidates for functional characterization. Though the association was not significant, we did observe modest evidence for increased prevalence of antibodies to Pfs230 and Pfs48/45 following the malaria transmission season. The potential for boosting of vaccine-induced immune responses during natural infection would enhance vaccine efficacy in the field (57). The extent and implications of this boosting are yet

to be explored, presenting an interesting question for future studies. Heterogeneity between studies remained significant even after assessing study-specific and methodological variation thus limiting our ability to describe a clear picture of the dynamics of NAI to sexual stages.

Limitations

As parasite detection in all the studies was by microscopy, with few studies also reporting PCR prevalence, we were restricted to using microscopy-based parasite prevalence for our analysis. The insensitivity of microscopy for parasite detection (58, 59) would lead to an underestimation of parasite prevalence. Better estimates of parasite prevalence may offer novel insights into the association between parasite prevalence and seroprevalence to sexual stage antigens improving our understanding of the dynamics of NAI in relation to sexual stage immunity. Furthermore, few studies currently report antibody titer in addition to prevalence though titer has been shown to positively correlate with transmission-reducing activity (23, 60, 61). Information on antibody titer can help generate useful information on the impact of naturally acquired sexual-stage immunity on malaria transmission and this should be considered in future studies.

Another limitation was that our search revealed only a few studies looking at naturally acquired immunity to sexual stage antigens which could limit the generalizability of our results. Study designs were also varied between studies complicating attempts to pool the studies in a meta-analysis to arrive at an overall seroprevalence estimate. While we were able to identify potential sources of heterogeneity in a univariable analysis, we could not further test the combined significance of the associated variables.

Conclusions

The combined analysis presented here showed that antibody responses to sexual stage antigens are identified in most studies conducted, suggesting that immunity to Pfs230 and Pfs48/45 is acquired following exposure to malaria parasites. Variability in study design and methodological differences contributed to significant between-study heterogeneity that was not fully addressed by the variables we examined in this analysis. This highlights the importance of harmonized protocols for carrying out and reporting seroepidemiological studies to enable comparability across different settings. Agreed guidelines for reporting seroepidemiological studies would be valuable for sexual antigens and could be based on the comprehensive set of criteria, MIOS, proposed by Fowkes et al. (56).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: doi: 10.5061/dryad.8bp05, doi: 10.5061/dryad.v60jk42, doi: 10.1038/nature24059.

AUTHOR CONTRIBUTIONS

MM, MK, and AB developed the protocol for the systematic review and meta-analysis. MM identified studies for review, carried out the data extraction, analyzed the data, and wrote the manuscript. AK carried out data extraction, assisted with the statistical analysis, and participated in drafting of the manuscript. TB assisted with data collection and participated in drafting of the manuscript. AB participated in design of the study and drafting the manuscript. PB participated in design of the study, assisted with the statistical analysis, and participated in drafting the manuscript. MK conceived of the study, participated in its design, identified articles for review, and participated in drafting the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02480/full#supplementary-material

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Corrigendum: Immune Responses to Gametocyte Antigens in a Malaria Endemic Population—The African *falciparum* Context: A Systematic Review and Meta-Analysis

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A Corrigendum on

Immune Responses to Gametocyte Antigens in a Malaria Endemic Population—The African *falciparum* Context: A Systematic Review and Meta-Analysis
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In the original article, there were errors in **Tables 1**, **2** and **3**, and to the text. In **Table 1**, one of the two study sites from Amoah et al.'s study (Parasites & Vectors, 2018) was erroneously excluded from the table hence the study was presented as one site, the age range of study participants from the Stone et al. (Nature Communications, 2018) study site "Hauts-Bassins (Burkina Faso)" was mistakenly indicated as "2–74" instead of "5–14" years and the reference for Skinner et al. given as "35" instead of "33". These errors have been corrected and the amended table appears in this article.

Furthermore, owing to the omission of the study site from Amoah et al.'s study, the figures quoted in the text on the overall number of study sites and the total number of study sites reporting seroprevalence to Pfs230 and Pfs48/45 were incorrect. The total number of study sites for Pfs230 was given as "14" instead of "15" and for Pfs48/45 given as "14" instead of "13". Also, we inadvertently provided the total number of individual study locations ("23") instead of the study sites—based on administrative region—("17") when providing a summary of the 12 studies that we included in the analysis. In addition, the citation for "Amoah et al." was was incorrectly cited as "Acquah et al." in the **Results** section, sub-section **Pfs230**, **Seroprevalence**, **Paragraph 1**.

These errors have been corrected and amendments made to the relevant result sections, given below.

Results section, sub-section **Study Selection and Characteristics**, **Paragraph 1**:

"The 12 studies were carried out across 17 study sites, majority of which were in West Africa (Burkina Faso, Senegal, Gabon, Cameroon, Ghana, and Mali) with only one study site in East Africa (Tanzania) and two study sites in Southern Africa (Zimbabwe) (**Table 1**). Ten articles (from 15 study sites) measured responses to Pfs230 and nine articles (13 study sites) measured responses to Pfs48/45. Six studies were longitudinal studies spread over the malaria transmission season with all but one measuring responses to both Pfs230 and Pfs48/45. Studies predominantly used ELISA as the immunoassay with only one study measuring responses using protein microarrays."

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TABLE 1 | Characteristics of studies included in the systematic review and meta-analysis.

Study (Reference)	Year	Country	Region of study site[c]	Sample size	Age group (years)	Antigen detected	Seasonality tested (Y/N)	Assay	Seropositivity cut-off	Negative control[d]	Selective recruitment[e]
Amoah et al. (34)[a]	2018	Ghana (Abura)	Central	65	6–12	Pfs230	No	ELISA^{R}	2 SD	Naïve	No
Amoah et al. (34)[a]	2018	Ghana (Obom)	Greater Accra	75	6–12	Pfs230	No	ELISA^{R}	2 SD	Naïve	No
Lamptey et al. (35)	2018	Ghana	Greater Accra	338	2–65	Pfs230	Yes	ELISA^{R}	3 SD	Test sample	No
Stone et al. (20)[b*]	2018a	Burkina Faso	Hauts-Bassins	33	5–14	Pfs230 and Pfs48/45	No	ELISA^{R}	3 SD	Test sample	Yes
Stone et al. (20)[b*]	2018b	Burkina Faso	Centre-Nord	38	2–10	Pfs230 and Pfs48/45	No	ELISA	3 SD	Test sample	Yes
Stone et al. (20)[b*]	2018	Cameroon	Centre	140	5–16	Pfs230 and Pfs48/45	No	ELISA^{R}	3 SD	Test sample	Yes
Bansal et al. (42)	2017	Zimbabwe	Mashonaland Central	181	6–14	Pfs48/45	No	ELISA^{R}	2 SD	Naïve	No
Paul et al. (43)	2016	Zimbabwe	Manicaland	150	6–16	Pfs48/45	No	ELISA^{R}	2 SD	Naïve	No
Ateba-Ngoa et al. (44)[b]	2016	Gabon	Moyen - Ogooue	286	3–50	Pfs230 and Pfs48/45	No	ELISA^{R}	3 SD	Test sample	No
Jones et al. (19)[b]	2015	Burkina Faso	Nord	200	5–16	Pfs230 and Pfs48/45	Yes	ELISA^{R}	3 SD	Test sample	No
Jones et al. (19)[b]	2015	Ghana	Greater Accra	108	5–17	Pfs230 and Pfs48/45	Yes	ELISA^{R}	3 SD	Test sample	No
Jones et al. (19)[b]	2015	Tanzania	Tanga Region	202	3–15	Pfs230 and Pfs48/45	Yes	ELISA^{R}	3 SD	Test sample	No
Skinner et al. (33)[b]	2015	Mali	Koulikoro 3 and Bamako	225	2–25	Pfs230 and Pfs48/45	Yes	Microarray^{R}	2 SD	No Template	No
Miura et al. (45)	2013	Mali	Kayes 2	45	18–60	Pfs230	No	ELISA^{R}	3 SD	Naïve	No
Ouedraogo et al. (24)[b*]	2018	Burkina Faso	Centre-Nord	128	1–55	Pfs230 and Pfs48/45	Yes	Two-site ELISA^{Ge}	3 SD	Naïve	No
Ouedraogo et al. (16)[a]	2011	Burkina Faso	Centre-Nord	296	1–>20	Pfs230 and Pfs48/45	Yes	Two-site ELISA^{Ge}	2 SD	Naïve	No
Van der Kolk et al. (46)	2006	Cameroon	Centre	236	5–14	Pfs230 and Pfs48/45	No	Two-site ELISA^{Ge}	2 SD	Naïve	No

[a] *Seroprevalence data provided by authors upon request.*
[b] *Seroprevalence data calculated from data provided by original authors, or from data available on public repositories.*
[b*] *Citation also includes citation of repository from which data was retrieved.*
[c] *Administrative region of study site from which participants were drawn, this was used infer predicted parasite prevalence rates standardized in 2 – 10-year olds ($PfPR_{2-10}$) that was then used to assign transmission intensity at the time of sampling.*
[d] *Negative control refers to the comparator used to assign seropositivity in the immunoassay. Naïve – malaria naïve volunteers; Sample – a proportion of statistically – defined seronegative individuals; No template - a 'no DNA control' used to detect reactivity to the expression vector used to produce protein for the array.*
[e] *Selective recruitment refers to studies that only recruited parasite positive individuals for antibody measurements.*
[R] *Recombinant protein;* [Ge] *gametocyte extract.*
SD, standard deviation.

TABLE 2 | Univariable meta-regression analysis of factors influencing reported seroprevalence to Pfs230.

	No. of studies (No. of Sites)	Coefficient (β)	Lower CI	Upper CI	*p*-value*	Residual I^2	I^2 change (%)
Age							
Children (ref.)	10 (14)						
Adults	6 (6)	0.21	0.05	0.38	**0.04**	95.36	**2.09**
Asexual parasite prevalence	6 (10)	−0.001	−0.005	0.002	0.51	95.37	2.08
Gametocyte prevalence	4 (8)	−0.002	−0.004	0.001	0.38	92.54	4.50
Transmission intensity							
Mesoendemic (ref.)	7 (8)						
Hyperendemic	6 (7)	−0.06	−0.23	0.11	0.51	96.18	1.25

(Continued)

TABLE 2 | Continued

	No. of studies (No. of Sites)	Coefficient (β)	Lower CI	Upper CI	*p*-value*	Residual I^2	I^2 change (%)
Season							
Dry (ref.)	6 (9)						
Rainy	5 (7)	0.07	−0.12	0.27	0.51	96.24	1.19
Assay							
ELISA (ref.)	6 (11)						
Microarray	1 (1)	0.31	0.08	0.55	0.07	95.29	2.17
Two-site ELISA	3 (3)	0.12	−0.06	0.29			
Antigen							
Gametocyte extract (ref.)	3 (3)						
Recombinant protein	7 (12)	−0.06	−0.25	0.13	0.51	96.31	1.12
Antigen concentration+							
0.1 μg/ml (ref.)	3 (7)						
1 μg/ml	3 (4)	0.26	0.09	0.43	**0.04**	93.52	**3.98**
Seropositivity cut-off							
2 SD (ref.)	4 (5)						
3 SD	6 (10)	−0.22	−0.37	−0.06	**0.04**	95.16	**2.30**

**p-values adjusted using the Benjamini and Hochberg correction for multiple testing; values in bold $p < 0.05$.*
+ Antigen concentration was only tested for studies using recombinant protein as antigen source.
CI, confidence interval; SD, standard deviation.

TABLE 3 | Univariable meta-regression analysis of factors influencing reported seroprevalence to Pfs48/45.

	No. of Studies (No. of Sites)	Coefficient (β)	Lower CI	Upper CI	*p*-value	Residual I^2	I^2 change (%)
Age							
Children (ref.)	9 (13)						
Adults	4 (4)	0.07	−0.12	0.27	0.49	94.90	−0.18
Asexual parasite prevalence	4 (8)	−0.003	−0.006	0.0003	0.11	91.41	3.96
Gametocyte prevalence	4 (8)	−0.003	−0.005	−0.002	**0.003**	70.82	**25.24**
Transmission intensity							
Hypoendemic (ref.)	1(1)						
Mesoendemic	5 (6)	−0.47	−0.89	−0.06	0.11	93.91	0.87
Hyperendemic	5 (6)	−0.38	−0.80	0.04			
Season							
Dry (ref.)	4 (6)						
Rainy	6 (8)	0.07	−0.09	0.24	0.47	93.12	1.70
Assay							
ELISA (ref.)	5 (9)						
Microarray	1 (1)	0.36	0.15	0.56	**0.016**	91.99	**2.89**
Two-site ELISA	3 (3)	0.09	−0.07	0.24			
Antigen							
Gametocyte extract (ref.)	3 (3)						
Recombinant protein	6 (10)	−0.01	−0.19	0.17	0.91	94.91	−0.19
Antigen concentration+							
0.1 μg/ml (ref.)	3 (7)						
1 μg/ml	2 (2)	0.30	0.06	0.54	**0.043**	92.65	**2.20**
Seropositivity cut-off							
2 SD (ref.)	5 (5)						
3 SD	4 (8)	−0.26	−0.39	−0.12	**0.003**	91.38	**3.54**

**p-values adjusted using the Benjamini and Hochberg correction for multiple testing; values in bold $p < 0.05$.*
+Antigen concentration was only tested for studies using recombinant protein as antigen source.
CI, confidence interval; SD, standard deviation.

Results section, sub-section **Pfs230, Seroprevalence**, **Paragraph 1**:

"Ten studies from across 15 study sites in Africa analyzed immune responses to Pfs230. The range of seroprevalence estimates was quite wide, ranging from 6% reported by Stone et al. in Soumousso and Dande villages, Burkina Faso (20) to 72% reported by Amoah et al. (34) (**Figure 2**). Significant heterogeneity was observed between the studies ($I^2 = 97\%$; 95% CI: 96–98%; $p < 0.01$) therefore, a pooled prevalence estimate was not calculated."

Results section, sub-section **Pfs48/45, Seroprevalence**, **Paragraph 1**:

"A total of 9 studies carried out over 13 study sites measured immune responses to Pfs48/45. The range of seroprevalence estimates reported was 0% from Stone et al.'s study sites in Burkina Faso (20) to 64% reported by Paul et al. from their study in the Makoni district in Zimbabwe (43). As with Pfs230, there was significant heterogeneity between the studies, $I^2 = 96\%$ (95% CI: 95–97%), and hence no pooled estimate was calculated (**Figure 3**)."

Additionally, there were errors in **Table 2** and **Table 3**, regarding the values for the **No. of Studies (No. of Sites)** under the variable **Antigen**. For **Table 2**, the numbers were switched around for gametocyte extract and recombinant protein and hence the numbers for **Gametocyte extract** read "7 (12)" instead of "3 (3)" and under the variable **Recombinant protein** read "3 (3)" instead of "7 (12)." For **Table 3** one study was missing from the count hence the value for the No. of Studies (No. of Sites) under the variable **Recombinant protein** read "5 (10)" instead of "6 (10)." The corrected **Tables 2** and **3** appear in this article.

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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Host Immune Responses and Immune Evasion Strategies in African Trypanosomiasis

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Parasites, including African trypanosomes, utilize several immune evasion strategies to ensure their survival and completion of their life cycles within their hosts. The defense factors activated by the host to resolve inflammation and restore homeostasis during active infection could be exploited and/or manipulated by the parasites in an attempt to ensure their survival and propagation. This often results in the parasites evading the host immune responses as well as the host sustaining some self-inflicted collateral tissue damage. During infection with African trypanosomes, both effector and suppressor cells are activated and the balance between these opposing arms of immunity determines susceptibility or resistance of infected host to the parasites. Immune evasion by the parasites could be directly related to parasite factors, (e.g., antigenic variation), or indirectly through the induction of suppressor cells following infection. Several cell types, including suppressive macrophages, myeloid-derived suppressor cells (MDSCs), and regulatory T cells have been shown to contribute to immunosuppression in African trypanosomiasis. In this review, we discuss the key factors that contribute to immunity and immunosuppression during *T. congolense* infection, and how these factors could aid immune evasion by African trypanosomes. Understanding the regulatory mechanisms that influence resistance and/or susceptibility during African trypanosomiasis could be beneficial in designing effective vaccination and therapeutic strategies against the disease.

Keywords: African trypanosomes, immunity, immunosuppression, immune evasion, resistance, susceptibility

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INTRODUCTION

African trypanosomiasis is a disease caused by extracellular hemoprotozoan parasites that belong to the genus *Trypanosoma*. Trypanosomes are unicellular parasites that are equipped with flagella which help with their movement (1). The disease is associated with serious health and economic problems in the affected countries, and can be fatal if not properly treated (2, 3). The human form of the disease is caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* while the animal form of the disease is mostly caused by *Trypanosoma congolense*, *Trypanosoma vivax,* and *Trypanosoma brucei brucei.* Trypanosomiasis is a vector borne disease. The transmission of the parasites from one host to another occurs during a blood meal by several species of tsetse flies belonging to the genus *Glossina*. For both human and animal African trypanosomiasis, *Glossina morsitans*, *Glossina tachinoides, Glossina fuscipes, Glossina pallidipes, Glossina swynnertoni,* and

Glossina palpalis are some of the important vectors responsible for transmission due to their wide distribution in countries where the disease is endemic (4), and their presence is often used as a key predictor of the disease.

Although it has been estimated that 65 million people living in 36 countries in sub-Saharan Africa are at risk of contracting the disease, the number of reported cases per year dramatically reduced (∼10,000 new cases annually) in 2009 due to increasing efforts to combat the disease (5–7). In 2018, the number of reported cases further reduced to 977, an almost 90% decline in 10 years (8). However, the real number of cases may be grossly underestimated because the disease is mostly found in rural communities in the endemic areas, and it has been estimated that only about 10% of the affected people living in these areas are accounted for (9, 10). In other words, the majority of the cases remain either undiagnosed or unreported, suggesting that the disease impact and statistics could be worse than currently believed.

Human African trypanosomiasis affects both the young and old especially those that engage in farm-related activities in the endemic rural areas, although few reported cases have also been reported in urban areas (11). This is most likely related to favorable environmental conditions in rural areas that favor breeding of the insect vector. Woody vegetations are known to support tsetse fly abundance as the flies tend to rest on tree trunks during the hot humid day. Efforts toward eliminating the disease were almost successful in the 1960s, but because of political instabilities and accompanying poor surveillance, the eradication process was disrupted, which allowed the disease to re-emerge (12–15). Economically, the threat posed by African trypanosomiasis to animals is much more than that posed to humans (16). The disease has been linked to severe food and economic loss in the affected regions (2, 3).

ROUTES OF INFECTION IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

Natural infection with African trypanosomes starts with intradermal injection of parasites along with tsetse saliva into the mammalian host by an infected tsetse fly. Following the introduction of the parasite into the dermis, the parasites undergo several transformations at this site with associated inflammatory response resulting in the development of chancre (1). These events precede the entry of the parasites into the blood stream of the infected host. Human African Trypanosomiasis usually develops from hemolymphatic phase to meningo-encephalitic phase; the meningo-encephalitic stage is one of the hallmarks of the disease and is often associated with severe alteration in the sleep-wake cycle (17). Cerebral infections with African trypanosomes in animals have also been documented; while *T. congolense* and *T. vivax* rarely invade the central nervous system (CNS), *T. brucei brucei* has been recovered from cerebrospinal fluid (CSF) and are able to cause central nervous system (CNS) impairment (18). Although there has been a report of *T. congolense* being found in the CSF, this was speculated to be aided by a mixed infection with other species (19). The tsetse fly saliva has been proposed to enable the blood-feeding process, promotes parasite transmission, and possesses powerful immunomodulatory properties including skewing T helper cell responses and anti-/proinflammatory properties (20–23). In contrast to this, the overwhelming majority of experimental infection studies utilize the intraperitoneal infection route, which involves inoculation of blood stream forms directly into the peritoneal cavity. It is conceivable that the use of intradermal route in experimental African trypanosome infection would capture some of the series of early events that occur during a natural infection (24). Therefore, it is likely that the intraperitoneal route of infection (as is used during most experimental infections) may not clearly represent the early immune response that occurs during natural infection when the vector bites their human or animal hosts (24). Indeed, it has been shown that intradermal infection of experimental animals is able to induce the activation of some immune mediators that are distinct from those seen during intraperitoneal infection (24, 25). Therefore, the use both the intradermal and intraperitoneal infection routes of infection (when possible) in experimental African trypanosomiasis would be helpful in comparing the immune responses due to different infection routes. This would enable us to further identify the missing links that could help in the understanding of the host-parasite interactions that regulate disease outcome.

KEY COMPONENTS OF IMMUNE RESPONSE TO AFRICAN TRYPANOSOMES

Both the innate and adaptive components of the immune system play crucial roles in resistance to African trypanosomiasis. Although African trypanosomes are free living in the bloodstreams of their mammalian host, and are therefore direct targets of antibody-mediated destruction, experimental animal models of infection show that a full component of the immune system (innate and adaptive) are critical for the development of optimal resistance to the infection.

Macrophages

Macrophages are one of the most important cells that contribute to innate immunity to African trypanosomiasis. They are capable of influencing the adaptive immune response directly through antigen presentation or indirectly by secreting many effector molecules including cytokines. During infection with African trypanosomes, classically-activated macrophages have been shown to contribute to parasite clearance via the process of phagocytosis (26), as well as through the production of proinflammatory cytokines and nitric oxide (27–31). Macaskill et al. conducted a study to specifically examine the roles played by antibody, macrophage activation, and complement in parasite clearance using trypanosomes labeled with [75Se]-methionine. Their results showed that clearance of parasites from circulation was mostly dependent on antibody-mediated phagocytosis by liver macrophages (32).

Both classical and alternative macrophage activation occur during African trypanosomiasis, and their effects vary depending on the timing of their activations. Enhanced survival of

trypanosome-infected mice has been associated with the ability to switch from classically-activated macrophages (M1) at the early stages of infection to an alternatively-activated phenotype (M2) during the advanced stages of infection (33, 34). This switch from M1 to M2 macrophages is essential because if left unchecked, classically-activated macrophages produce excessive amounts of proinflammatory cytokines that induce immune hyperactivation, causing collateral tissue damage and death in trypanosome-infected animals (35, 36). Thus, the inability of mice to upregulate alternative macrophage activation while concomitantly downregulating classical activation during the advanced stage of *T. congolense* infection led to enhanced susceptibility to infection, which was associated with excessive production of proinflammatory cytokines and early death of infected mice (37).

Several studies have associated pathology—mostly anemia and cachexia—during African trypanosome infection with the over-activation of macrophages and the resulting release of harmful molecules such as TNF-α and nitric oxide (38, 39). However, the underlying mechanisms involved in this process are still not fully investigated. While TNF-α is directly linked to the development of anemia in *T. brucei* infection, it appears not to be involved in *T. congolense* infection. Instead a direct alteration of red blood cells has been linked. (38–42).

The roles of macrophages during intradermal infection have not been well-investigated. Wei et al. demonstrated that mice that are deficient in inducible nitric oxide synthase (iNOS) were susceptible to intradermal low dose *T. congolense* infection (24), and proposed that macrophages are at the center of innate control of primary intradermal infection. However, results from our lab have shown that depletion of macrophages before *T. congolense* infection did not alter the resistance of mice to primary intradermal infection (25). This observation suggests that host factors other than macrophages are responsible for mediating early resistance observed during intradermal infection. More studies are needed to fully determine the roles played by macrophages in enhanced resistance observed during intradermal infection.

Complement

Although African trypanosomes have different mechanisms to avoid lysis by complement, the activation of this important arm of the innate immune defense system is considered to be critical for mediating parasite clearance in infected animals. Both the classical and alternative pathways of complement system are activated during infection with African trypanosomes. Activation of the classical pathway is mediated by specific antibodies against the parasite and has been reported to contribute to parasite clearance via antibody-mediated lysis, opsonization and phagocytosis (43). The antibody-independent alternative pathway is usually activated during the early stages of infection when specific antibodies have not been formed, and has also been shown to contribute to parasite clearance (44).

Although phagocytosis of African trypanosomes by macrophages can occur *in vitro* in the absence of complement (45), the efficiency of parasite clearance and immune complex removal is greatly enhanced in the presence of complement (46). Indeed, it was demonstrated that depletion of the complement component C3 by treatment with cobra venom factor leads to significant reduction in hepatic uptake of opsonized trypanosomes (32). However, other studies showed that partial depletion of C3 did not affect either parasitemia control of *T. brucei brucei* or phagocytosis of *T. brucei rhodesiense* (47). Jarvinen and Dalmasso found that parasite control could not be attributed to the presence of C3, C5, or late acting complement factors (48). In addition, Jones and Hancock found no marked differences in the survival periods of C5-deficient and C5-sufficient mice infected with African trypanosomes (49). Collectively, these findings suggest that complement activation may be required but not critical for resistance to African trypanosomes. However, given that the cleavage products of C3 and C5 (C3a and C5a) have been reported to help in the initiation of inflammatory responses during infection (50), and that alternative and classical complement activation is higher in the relatively resistant C57BL/6 mice following *T. congolense* infection (51, 52), it is conceivable that complement activation may contribute to optimal resistance to certain species of African trypanosomes.

T Cells

Both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells play a critical role in regulating the outcome of infection with African trypanosomes. $CD4^+$ T cells contribute to resistance by producing cytokines that regulate other innate and adaptive immune cells, and by providing help to B cells to ensure efficient isotype class-switching and production of specific antibody responses to parasite antigens. $CD4^+$ T helper cells provide signals that regulate B cell survival and differentiation into antibody producing cells (53). In support of this, Shi et al. showed that anti-parasite IgG2a production, as well as IFN-γ and IL-10 levels, was impaired in *T. congolense*-infected $CD4^+$ T cell deficient mice (54). Following this observation, Tabel et al. proposed that future vaccines against African trypanosomiasis should be targeted toward encouraging the generation of T helper 1 cells (Th1) that would support B cells in class-switching from IgM to IgG2a during infection (55). Interestingly, it was found that $CD4^+$ T cell deficient mice infected with *T. congolense* had significantly lower parasitemia and prolonged survival period compared with their WT mice, suggesting that $CD4^+$ T cells might also contribute to disease pathogenesis and death in infected mice (54).

The role of $CD8^+$ T cells has been controversial in African trypanosomiasis. Earlier studies suggested that polyclonal activation of $CD8^+$ T cells by *T. brucei brucei*-derived T lymphocyte triggering factor (TLTF) leads to massive release of IFN-γ, which is responsible for profound immunosuppression and susceptibility to the infection (56, 57). However, a study by Wei and Tabel showed that the beneficial effect of anti-CD25 treatment in mice during *T. congolense* infection was lost upon depletion of $CD8^+$ T cells (58), suggesting that $CD8^+$ T cells may be playing a protective role during *T. congolense* infection. In another study that investigated the relative contributions of $CD4^+$ and $CD8^+$ T cells in *T. brucei* infection, Liu et al. (59) reported that IgG antibody synthesis was dependent on $CD4^+$ T cells and not $CD8^+$ T cells. In addition, they showed that infected $CD8^+$ T cell-deficient

mice had lower parasitemia and survived significantly longer than their WT counterpart mice. However, this enhanced survival was lost upon depletion of $CD4^+$ T cells. Furthermore, cytokine (IFN-γ and IL-10) production during infection was also attributed to $CD4^+$ but not $CD8^+$ T cells (59). These observations indicate that $CD8^+$ T cells mediate susceptibility while $CD4^+$ T cells mediate protection during infection with *T. brucei brucei*.

Our understanding of the roles played by T cells has been simplified by the Th1 (proinflammatory properties) and Th2 (anti-inflammatory properties) paradigm. Overall, the control of parasites during infection with African trypanosomes is believed to be associated with a Th1 response during the early phase of infection and a switch to a Th2 phenotype in the advanced stage of infection (33, 34). In support this, *T. congolense*-infected $TSLPR^{-/-}$ mice with impaired Th2 response are highly susceptible to *T. congolense* infection during the chronic stage of the disease, and died significantly earlier than their WT controls (37). This susceptibility was associated with increased production of proinflammatory cytokines by $CD4^+$ T cells from these mice, which was reversed upon treatment of the infected $TSLPR^{-/-}$ mice with anti-IFN-γ monoclonal antibody (37).

B Cells

Because African trypanosomes are extracellular bloodstream parasites, they are constantly exposed to humoral immune factors and are direct target for antibody-mediated destruction. Indeed, B cell deficient mice are highly susceptible to African trypanosomes (60). In addition, passive transfer of variant-specific antibodies or B cells (but not T cells) to immunocompromised mice results in variant-specific protection (60, 61). Furthermore, the differential resistance observed in several strains of mice and cattle following infection with African trypanosomes has been attributed to differences in the production of parasite-specific antibodies (62–64). A study using mice that lack Bam32 further demonstrated the importance of strong B cell response for protection against infection with African trypanosomes (65). Bam32 is a B cell adaptor protein that plays a critical role in B cell activation (66), survival (67), and antigen presentation (68). Bam32 deficient mice on a relatively resistant background were more susceptible than their WT counterpart mice to *T. congolense* infection and showed impaired germinal center response as well as significantly low levels of parasite-specific IgG antibodies in the serum (65, 68).

B cells become activated upon encountering their cognate antigens, and this is followed by the initiation of germinal center formation with the help of follicular $CD4^+$ T helper cells (Tfh) (69). Germinal centers are large areas in the secondary lymphoid organs where intense B activities such as proliferation, somatic hypermutation, selection, and class switch recombination take place, resulting in the production of various antibody isotypes with high antigen binding affinity (53). The requirement of B cells during infection with African trypanosomiasis centers on optimal activation, efficient germinal center (GC) formation, and production of strain-specific antibodies.

One of the hallmarks of African trypanosomiasis is excessive polyclonal activation of B cells leading to increased serum levels of trypanosome-specific and non-specific antibodies, including heterophilic and autoantibodies (70, 71). These observations led to the postulation that African trypanosomes possess molecules (VSG for instance) that could non-specifically activate B cells to produce antibodies (70). During infection with African trypanosomes, both T cell-dependent and T cell-independent antibody response are produced against the variant and invariant VSG molecules, cytoplasmic, and other nuclear parasite antigens (72). However, the overall quality (as assessed by binding affinity, isotype, and quantity) of the response is increased in the presence of T cells (72). For example, although IgM anti-VSG antibodies (which are mostly produced in a T cell-independent manner) can mediate parasite clearance, the different subclasses of IgG antibodies, whose production are T cell dependent, mediate a more effective parasite clearance in both mice and cattle compared to IgM (62, 73).

Cytokines

The profile and magnitude of cytokines produced during African trypanosomiasis play a critical role in determining susceptibility and resistance to the disease. Although the contributions of some cytokines in the pathogenesis of African trypanosomiasis have been demonstrated in different experimental settings, it is challenging to fully determine the precise role of specific cytokines in disease pathogenesis because of their pleiotropic activities (55). Studies have shown that infection with African trypanosomes leads to massive production of proinflammatory cytokines in the infected mammalian host. The initial inflammatory response during infection usually leads to the release of proinflammatory mediators like TNF-α, IL-1, IL-6, and NO by classically activated macrophages, and these have been shown to play important roles in mediating early protection during infection (26, 30, 74–78). Other cytokines like IL-12, MCP-1, IL-10, IFN-γ, and IL-4 have also been shown to mediate either pro-inflammatory or anti-inflammatory activities during infection (26, 54, 79–83).

Although the initial outburst of inflammatory cytokines is essential for resistance, it requires regulation to prevent collateral tissue damage. The switch from classically activated to alternatively activated macrophages later during infection by type II cytokines such as IL-4, IL13, and TSLP is critical for maintaining a Th2 type environment which have anti-inflammatory properties (1, 37). In fact, protection during infection with African trypanosomes is associated with the ability to switch from an early Th1 to Th2 response during the later stages of infection (33, 34, 37, 84). In line with this, the absence of TSLP signaling (which is a key cytokine that drives Th2 differentiation), in *T. congolense*-infected mice led to the inability to control more than two waves of parasitemia and early death compared to their wild-type counterpart mice (37). This inability to effectively control parasitemia was associated with overproduction of proinflammatory cytokines (including IFN-γ and TNF-α) and impaired activation of alternatively activated macrophages (37).

The effect of cytokines in the pathogenesis of African trypanosomiasis is complex and depends on the quantity and time of production during the infection (55). For example,

while the production of IFN-γ and TNF-α are critical for protection during infection with African trypanosomes (85–88), their production in excessive amounts is detrimental and leads to susceptibility and death of infected mice (27, 79, 82). For instance, while IFN-γ-deficient mice are highly susceptible to *T. brucei* or *T. congolense* infection and fail to control the first wave of parasitemia (85, 87), neutralization of IFN-γ by antibody treatment during *T. congolense* infection is associated with enhanced resistance (lower parasitemia and prolonged survival) of the highly susceptible mice (79). Furthermore, acute death of infected relatively resistant mice following treatment with anti-IL-10 receptor antibody was completely abrogated by co-treatment with anti-IFN-γ mAb (82). These observations suggest that IFN-γ is a key mediator of death in these mice.

Another key cytokine that regulates the outcome of infection with African trypanosomes is IL-10. Due to its anti-inflammatory properties, IL-10 acts to downregulate excessive effector activities of both T cells and macrophages (82, 89), which are key cells that are involved in the production of inflammatory cytokines following infection with African trypanosomes. In *T. congolense*-infected cattle, reduced nitric oxide production and increased IL-10 and IL-4 mRNA levels were linked to protection (73, 90).

IMMUNE EVASION STRATEGIES USED BY AFRICAN TRYPANOSOMES

Antigenic Variation

Adaptation mechanisms within the host are known to exist among bacteria, parasites, and viruses. Antigenic variation is one of the hallmarks of African trypanosomes and constitutes a major adaptation mechanism of evading their host immune response. In fact, it has been suggested that the inability to fully understand the mechanisms that regulate antigenic variation during infection with African trypanosomes is one of the major obstacles standing in the way of developing a vaccine for African trypanosomiasis (38).

Upon injection into the skin, the parasites grow and multiply in the bloodstream of their mammalian host. Because African trypanosomes are completely extracellular in nature, they are continuously exposed to the host's humoral immune defenses. Although clone-specific antibodies are effective in mediating parasite clearance (62, 85), the natural ability of the parasites to undergo antigenic variation during the course of infection renders the antibodies ineffective at mediating cure. The expression of new VSG allows the parasites to evade antibody-mediated destruction thus permitting them to grow and multiply, and requiring the host to initiate new antibody responses against the emerging new clones.

Bloodstream forms of African trypanosomes are covered with a densely packed protective coat comprising of over 10^7 copies of variant surface glycoprotein (VSG) molecules (1). These millions of identical glycoprotein molecules function to prevent complement-mediated lysis of the parasites (91, 92). They are attached to the surface of the plasma membrane of the parasite via the glycosylphosphatidylinositol (GPI) anchor (93). Although mostly membrane bound, soluble forms of VSG are released into the circulation upon cleavage of the GPI anchor by parasite-encoded phospholipase C (PLC) known as GPI-PLC (94). This process has been reported to trigger some inflammatory responses during infection (95).

Trypanosomes contain hundreds of VSG genes (constituting about 10% of the entire parasite genome) with only about 7% being fully functional (96). The transcription of VSG genes occurs at a telomere of the chromosomes containing the VSG expression sites (97). Because only one expression site can be active at any given time, only one of the VSG molecules is expressed on the surface coat of the parasite leading to identical display of surface coats (1). In addition, because antibody response is made only to this particular antigenic type that is being expressed, a switch in VSG expression would lead to the initiation of new antibody response, a condition that could subsequently pave the way for immune exhaustion due to the continuous need to mount immune response to numerous VSG-expressing clones. Trypanosomes are able to control VSG gene expression by turning off an active expression site (and turning on a previously silent expression site) and by rearrangement of the VSG genes mostly by reciprocal recombination and gene conversion (98–100). Efforts targeted at disrupting the VSG switching by the parasites could be a major step in designing effective disease control strategies.

Polyclonal B Cell Activation

Because of their extracellular life style, B cells play a critical role in clearance of African trypanosomes from the blood of infected hosts by producing parasite-specific antibodies. As a result, they have developed mechanisms to suppress and evade the host specific antibody responses. Trypanosomes are able to exploit the ability of B cells to produce antibodies and use this to induce excessive activation of antibody-producing cells. This often leads to an increase in plasma levels of specific and non-specific immunoglobulins in infected animals. This hypergammaglobulinemia, which results from extensive B cell expansion, was first reported as a major feature of infection with African trypanosomes (101). While specific antibodies against parasite antigens are produced in both T cell-dependent and independent pathways (72), the induction of non-specific (polyclonal) B cell activation in a T cell-independent manner represents a strategy through which the parasites regulate specific antibody responses that contributes to immune evasion (102). Although not clearly known, it has also been reported that experimental *T. congolense* infection causes B cell depletion in the bone marrow as well as the periphery, which in turn impact negatively on the specificity of anti-VSG immunoglobulins produced during infection (103). The resulting impaired B cell development and maturation makes it impossible for the optimal development of memory B cell subsets that are key when considering vaccination strategies (104).

Several trypanosomal moieties, including DNA and VSG have been shown to contribute to polyclonal B cell activation following infection with African trypanosomes. Trypanosomal DNAs are able to initiate TLR-9 signaling leading to non-specific B cell activation and production of poly-specific antibodies to VSG (102, 105). Indeed, autoantibodies against red blood cells,

cardiolipids, nucleic acids, rheumatoid factors, and component of CNS myelin (the galactocerebrosides) have been detected in sera of infected animals (106–111). Furthermore, the overproduction of antibodies could result in over-engagement of the Fcγ receptors on phagocytes, which would eventually contribute to impaired opsonization and phagocytosis of parasites (102, 105). In addition, an increased in a sub-population of CD5 expressing B cells have also been documented, and these cells are believed to be responsible for the excessive production of immunoglobulins and autoantibodies during *T. congolense* infection in cattle (105).

Hypocomplementemia

African trypanosomes are able to take advantage of complement activation to evade the host immune response. Because the classical complement activation is critical for lysing parasites coated with VSG-specific antibodies, trypanosomes, in an effort to enhance their survival, are able to shed enormous VSG in the circulation which leads to the formation of immune complexes with antibodies. This "decoy mechanism" of shedding VSG prevents the deposition of membrane attack complex on the parasite cell membrane, thereby preventing complement-mediated lysis of the parasite. The continuous activation of the complement system often leads to a state of hypocomplementemia, which is a hallmark of infection with African trypanosomiasis (112). Also, due to their spatial and dense arrangement, the VSG is known to mask the binding of complement proteins on the plasma membrane leading to the inability to trigger alternative complement activation. This results in halting of the alternative pathway activation at the C3 convertase stage thereby preventing further activation of C5–C9 stages and subsequent formation of the membrane attack complex that usually initiate lysis of trypanosomes (113, 114).

Induction of Regulatory Cells

One of the hallmarks of African trypanosomiasis is suppression of lymphocyte proliferation, and this has been reported to be one of the key factors that prevent parasite control in infected animals (115–117). Several cell types have been implicated in the pathogenesis of immunosuppression in African trypanosomiasis. Although suppressor macrophages and suppressor T cells were the earliest identified culprits (118–120), recent studies have clearly revealed additional roles played by regulatory T cells (25, 58, 121) and myeloid-derived suppressor cells (122). Schleifer and Mansfield (123) showed that macrophages suppress T cell responses through the production of reactive nitrogen intermediates and prostaglandins. Similarly, independent studies (using anti-CD25 mAb treatment) have shown that $CD4^+CD25^+Foxp3^+$ T cells (Tregs) play a role in suppression of immune response to *T. congolense* (25, 58, 121). In these studies, immunity to the parasites (including control of parasitemia and survival) was enhanced in the absence of Tregs. Indeed, it was found that during intradermal infection with *T. congolense*, the parasites induces the expansion of regulatory T cells as a mechanism to induce immune suppression in order to evade host's immune response and enhance their survival (25).

Suppressor macrophages have also been shown to mediate suppression of host immune response as a means to enhance parasite survival. For example, trypanosomes are able to take advantage of the alternative macrophage activation to enhance their survival in the host. These parasites have been shown to preferentially induce alternative macrophage activation with the sole aim of upregulating host arginase, which has been shown to reduce the synthesis of trypanocidal nitrosylated compounds as well as upregulate L-ornithine production, a critical process in the synthesis of polyamines that is required for parasite growth (124, 125). Recently, we showed that *T. congolense* infection was associated with the induction of myeloid-derived suppressor cells, which suppressed T cell responses (proliferation and IFN-γ production) during *T. congolense* infection in an arginase-1-dependent mechanism (122). This suggests that targeting the arginase pathway during infection could limit parasite survival by increasing various effector responses directed against the parasites.

Trypanosome Lytic Factors (TLFs)

Two species of African trypanosomes, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, are able to infect their human host. This is due to the parasites' ability to manipulate and evade the effect of TLFs that are known to have anti-parasite activity. TLF comprises of two serum complexes, Trypanosome Lytic Factor 1 and 2 (TLF1 and TLF2) (126, 127). These complexes are made up of primate-specific protein haptoglobin-protein (HPR) and apolipoprotein L1 (APOL1) (128). It is known that HPR is responsible for targeting TLF1 to the parasites, while APOL1 is the trypanolytic toxin (129–131). In *Trypanosoma brucei rhodesiense,* resistance to APOL1 is related to the expression of an expression site-associated gene (ESAG) generally referred to as serum resistance-associated (SRA) gene (132, 133). SRA is able to bind to APOL1, and the resulting interaction leads to the inactivation of APOL1 via the inhibition of its membrane insertion, resulting in degradation by proteases (128, 134). In *Trypanosoma brucei gambiense,* resistance is conferred through *T. b. gambiense*-specific glycoprotein (TgsGP) (135). The deletion of the gene that encodes TgsGP renders the parasite susceptible to normal human serum (136, 137). TgsGP inhibits APOL1 toxicity though a hydrophobic β-sheet (136) that initiates membrane stiffening, which could inhibit APOL1 membrane insertion leading to APOL1 degradation by endosomal proteases (128).

Trypanosome-Suppressive Immunomodulating Factor (TSIF)

African trypanosomes (specifically *T. brucei*) encodes a suppressive protein, Trypanosome-suppressive immunomodulating factor (TSIF) that has been shown to have immunosuppressive actions *in vivo* (138). Treatment of mice with recombinant TSIF resulted in the activation macrophages that predominantly produce TNF-α and NO, suggesting that this protein favors the classical activation of macrophages (138). TSIF has also been shown to significantly impair Th2-induced inflammation in allergic asthma model, which further strengthens its ability to preferentially induce a Th1 inflammatory condition (139). Although, classical macrophage activation could be important for trypanosome

clearance during the early phase of infection, a switch to a more alternatively-activated macrophage phenotype toward the late stage infection is required to prevent excessive inflammation and death of infected mice (33, 34, 37, 84). By employing the use of TSIF, trypanosomes are able to manipulate the host immune system by sustaining the classical macrophage activation during infection thereby suppressing the development of alternatively activated macrophages (Th2 condition). This could lead to exacerbated inflammation and death of infected animals.

CONCLUDING REMARKS

There is currently no vaccine against African trypanosomiasis and the current drugs for treatment of the disease are relatively ineffective. In addition to being toxic and expensive, the effectiveness of the current drugs is further hampered by increased risk of drug resistance and disease relapse (140–142). The development of a vaccine against this disease has been made virtually impossible due to antigenic variation and the difficulty in understanding the factors that regulate this and other important and highly complex host immune evasion mechanisms (38). Given that decades of research have failed to develop a vaccine against African trypanosomiasis, it is imperative that current research strategies have not yielded meaningful information regarding the induction and maintenance of effective and/or protective immune response to these parasites (143). Therefore, a change in strategy and our current way of thinking (including experimental designs and animal models) is needed. Studies focusing on halting various events that contribute to impaired immune response and immune evasion strategies by the parasites (**Figure 1**) could represent a major pathway toward disease control.

Because using the intraperitoneal route of infection does not mimic or represent the activities that take place during natural infection (which is initiated via intradermal inoculation by the tsetse fly), a switch to a more natural intradermal route of infection could be more beneficial in understanding key immune responses to these parasites, and could provide helpful information regarding vaccine development (25, 144). Furthermore, the causes of polyclonal B cell activation need to be further investigated since the parasite's ability to induce non-specific B cell responses to enhance their survival in the host still poses a great challenge in vaccine discovery. Although data showing the importance of optimal germinal center reaction during infection are beginning to appear (65), similar studies aimed at understanding other factors that regulate the parasite's ability to induce non-specific antibody responses could be beneficial. We showed that the ability of $CD4^+$ T cells to proliferate and produce IFN-γ during *T. congolense* infection was inhibited by myeloid-derived suppressor cells in an arginase-1-dependent manner (122). This identifies arginase-1 as a potential target in African trypanosomiasis when considering treatment and vaccination strategies (144). It would be highly interesting to determine whether these exciting and novel mechanisms of immune regulation during *T. congolense* infections operate during infection with other African trypanosome species, particularly those that cause disease in humans.

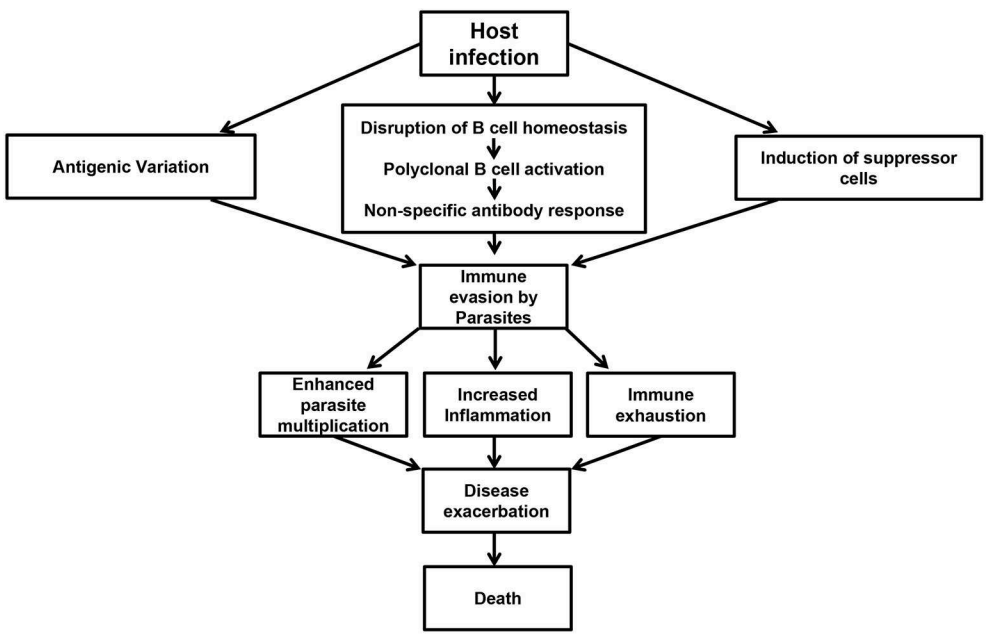

FIGURE 1 | Events preceding immune evasion by parasites. Following infection, trypanosomes are able to undergo antigenic variation, disrupt B cell homeostasis, and initiate polyclonal B cell activation leading to the production of non-specific antibody responses. In addition, the infection is also associated with the induction of suppressor cells like Tregs and MDSCs. These events contribute to immune evasion by the parasites resulting in poor parasite control, increased inflammation, immune exhaustion, disease exacerbation, and death in untreated animals.

In addition to the ongoing search for anti-parasite vaccine, adopting the anti-disease vaccine approach (145) could also serve as a great option to explore in African trypanosomiasis. This is based on the fact that trypanosome-associated factors could be targeted and nullified, which in turn would limit pathologies that are the major cause of disease. In line with this, cysteine peptidases have been identified as a potential candidate for anti-disease vaccine target (146) as they have been proposed to be likely involved in inducing alterations and pathologies that result in immunosuppression, anemia, and CNS disorder (147–150). Also, because the VSG GPI anchor induces very strong proinflammatory cytokine response that contributes to disease and pathologies (151, 152), it has been suggested that the GPI could be a promising target for anti-disease vaccine development (153). Indeed, it has been shown that GPI-based treatment alleviates trypanosomiasis-associated immunopathology, including anemia, weight loss, liver damage, inflammation, and proinflammatory cytokine production resulting in prolonged survival (154, 155). Although the efficacy of using synthetic GPI as vaccine is yet to be evaluated in infections caused by African trypanosomes, a study conducted in a mouse model of severe malaria using chemically-synthesized *Plasmodium falciparum* GPI glycan for immunization led to a significant reduction in early death and malaria-associated pathologies during challenge infection (153). This was associated with *in vitro* antibody (against GPI)-dependent neutralization of *P. falciparum*-induced inflammation (153). It would be interesting to investigate the impact of vaccination with synthetic GPI in the pathogenesis of experimental African trypanosomiasis, including the production of disease-enhancing proinflammatory cytokines.

AUTHOR CONTRIBUTIONS

CO: literature search and drafting. JU: provided corrections to the draft.

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TLR-2 and MyD88-Dependent Activation of MAPK and STAT Proteins Regulates Proinflammatory Cytokine Response and Immunity to Experimental *Trypanosoma congolense* Infection

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It is known that *Trypanosoma congolense* infection in mice is associated with increased production of proinflammatory cytokines by macrophages and monocytes. However, the intracellular signaling pathways leading to the production of these cytokines still remain unknown. In this paper, we have investigated the innate receptors and intracellular signaling pathways that are associated with *T. congolense*-induced proinflammatory cytokine production in macrophages. We show that the production of IL-6, IL-12, and TNF-α by macrophages *in vitro* and *in vivo* following interaction with *T. congolense* is dependent on phosphorylation of mitogen-activated protein kinase (MAPK) including ERK, p38, JNK, and signal transducer and activation of transcription (STAT) proteins. Specific inhibition of MAPKs and STATs signaling pathways significantly inhibited *T. congolense*-induced production of proinflammatory cytokines in macrophages. We further show that *T. congolense*-induced proinflammatory cytokine production in macrophages is mediated via Toll-like receptor 2 (TLR2) and involves the adaptor molecule, MyD88. Deficiency of MyD88 and TLR2 leads to impaired cytokine production by macrophages *in vitro* and acute death of *T. congolense*-infected relatively resistant mice. Collectively, our results provide insight into *T. congolense*-induced activation of the immune system that leads to the production of proinflammatory cytokines and resistance to the infection.

Keywords: African trypanosome, immune response, proinflammatory cytokines, MAPK, STAT

INTRODUCTION

African Trypanosomiasis, an infectious disease of humans and animals, is caused by various species of protozoan parasites belonging to the genus *Trypanosoma*. Because of minimal research in treatment and control measures, it is considered as one of the neglected tropical diseases similar to other parasitic diseases, such as Schistosomiasis, Leishmaniasis, Chagas disease, etc. (1).

African animal trypanosomiasis is primarily caused by *Trypanosoma* (*T.) congolense, T. vivax, and T. brucei brucei* and presents as a mild disease in wild animals but very fatal in domestic animals if untreated. The disease has severe economic impact and adversely affects livestock production and farming in the affected regions of sub-Saharan Africa. *Trypanosoma congolense* is the most important African trypanosome and causes debilitating acute and chronic disease in cattle and other domestic animals. Because the parasites are purely extracellular but intravascular, they are unable to leave the circulation and are constantly exposed the to the host's immune system. As a result, they have developed sophisticated evasion mechanisms including antigenic variation of the variant surface glycoprotein (VSG) (2, 3), polyclonal B-lymphocyte activation (4), and induction of immunosuppression (5–7). Mice are the most common animal models for experimental African trypanosomiasis and have provided great insight into the immunopathogenesis of the disease. BALB/c mice are highly susceptible to experimental *T. congolense* infection because they are unable to control the first wave of parasitemia and die within 8–10 days. On the contrary, C57BL/6 mice are relatively resistant to infection and control several waves of parasitemia and survive for over 100 days (8). It has been shown that death of infected animals is due in part to hyper-activation of immune cells (particularly macrophages and T cells) resulting in excessive production of pro-inflammatory cytokines (including IFN-γ, IL-6, IL-12, and TNF), which leads to systemic inflammatory response like syndrome (8). However, the innate receptors, adaptor proteins and signaling pathways associated with *T. congolense*-induced cytokine production in macrophages are not known.

Toll like receptors (TLRs) are a family of type 1 transmembrane receptors found on innate cells and play critical role in the initiation of innate immune response. Ligation of these receptors by their ligands leads to activation of various signal transduction pathways that lead to the induction of various genes including inflammatory cytokines. TLRs have an important role in recognition of molecular signatures of microbial infection, propagation of various signaling pathways, and directing the adaptive immune response (9). The extracellular leucine-rich repeats of TLRs are responsible for recognition of pathogens while the cytoplasmic region, known as Toll/interleukin-1 receptor (TIR) domains, are responsible for initiating intracellular signaling events (10). This involves heterophilic interactions of TIR domain with cytosolic adaptor proteins, such as myeloid differentiation of primary response protein 88 (MyD88), TIR-domain containing adapter protein (TIRAP), TIR domain-containing adapter inducing IFNβ (TRIF), and TRIF-related adaptor molecule (TRAM) (10, 11). However, MyD88 is the major adaptor protein that is a part of almost all TLR signaling pathways. Individual TLRs interact with different combinations of adaptor proteins resulting in the activation of various transcription factors, such as nuclear factor (NF)-κB, activating protein-1 (AP-1), and induces a specific immune response. Members of both mitogen-activated protein kinases (MAPKs) and signal transducer and activator of transcription (STAT) family members can interact to activate multiple intracellular signaling pathways that lead to increased cytokine production.

In this study, we investigated the innate receptors involved in *Trypanosoma congolense* recognition in macrophages, the role of MyD88, and the intracellular signaling molecules involved in *T. congolense*-induced cytokine production. We show that *T. congolense*-induced cytokine production is dependent on MyD88-mediated activation of MAPKs (ERK, p38, JNK) and STATs (STAT1 and STAT3) signaling and that TLR2 is the critical receptor involved in parasite recognition, production of proinflammatory cytokines, and induction of resistance in infected mice.

MATERIALS AND METHODS

Mice

Six to eight weeks old female C57BL/6 and outbred Swiss white (CD1) mice used in this study were purchased from Charles River, St. Constante, Quebec, Canada. MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were purchased from The Jacksons Laboratory (Bar Harbor, ME). Animals were housed at the Central Animal Care Services (CACS) facility, University of Manitoba, Winnipeg, Canada. The studies were approved by the University of Manitoba Animal Care Committee and carried out in accordance with the regulation of the Canadian Council on Animal Care.

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from DIFCO Laboratories (Detroit, MI). Rabbit anti-mouse p38 and ERK 1/2 mAbs, affinity-purified rabbit anti-phospho p-38, affinity purified mouse anti-phospho ERK 1/2, rabbit anti-total and phosphor-specific SAPK/JNK mAbs, rabbit polyclonal anti-STAT1, rabbit polyclonal anti-STAT3, and rabbit anti-phospho and total NF-κB mAb were purchased from Cell Signaling Technology (Danvers, MA). The p38 MAPK inhibitor 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB-203580), p42/44 ERK inhibitor 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene (U-0126), and JNK inhibitor anthra[1,9-*cd*]pyrazol-6(2*H*)-one, 1,9-pyrazoloanthrone (SP 600125) were purchased from Calbiochem (Mississauga, Ontario, Canada). Fludarabine (specific inhibitor of STAT-1) was obtained from Sigma-Aldrich (Mississauga, Ontario, Canada). STAT3 inhibitor, 2-Hydroxy-4-(4-methylphenyl) sulfonyloxy)acetyl)amino)-benzoic acid (S31-201) was obtained from Santa Cruz Biotechnology (Dallas, TX).

Infection and Estimation of Parasitemia

Trypanosoma congolense (Trans Mara Strain), variant antigenic type (VAT) TC13 was used in this study (12). Frozen TC13 stabilates were expanded in immunosuppressed (treated with cyclophosphamide) CD1 mice as previously described (12). After 3 days of infection, blood was collected from CD1 mice by cardiac puncture. Parasites were purified from blood using DEAE-cellulose anion-exchange chromatography (13), washed and resuspended in Tris-saline glucose (TSG) solution containing 10% heat-inactivated FBS (TSG-FBS) at a final concentration of

10^4/ml. Mice (WT, MyD88$^{-/-}$ and TLR2$^{-/-}$) were infected by intraperitoneal injection of 100 μl TSG-FBS parasite suspension (containing 10^3 parasites). Daily parasitemia was determined by counting the number of parasites in a drop of the blood using a microscope as previously described (14). Briefly, a drop of blood (taken from the tail vein of infected mice) on a microscopic slide was covered with a cover slip and the numbers of parasites present in at least 10 fields were counted at 400× magnification.

Preparation of Trypanosomal Whole Cell Extract (WCE)

To prepare *T. congolense* whole cell extract (WCE), isolated parasites were resuspended in TSG at a final concentration of 10^8/ml and then subjected to 3–5 sonication cycles (5 min per cycle). Thereafter, the sonicate was further subjected to freeze/thawing (at −80°C) up to about 8 cycles (30 min/cycle), aliquoted and stored at −80°C until used. Endotoxin level in WCE preparations was determined by the LAL kit (E-TOXATE, Sigma) according to the manufacturer's suggested protocol. Endotoxin level was <0.05 EU/ml.

Cell Lines, Bone Marrow-Derived Macrophages (BMDM), and *in vitro* Cell Cultures

The origin of ANA-1 cells or retrovirus-immortalized bone marrow-derived macrophage cell lines from C57BL/6 mice has been described previously (15). The immortalized cell lines were grown in complete RPMI medium (RPMI 1640 medium supplemented with 10% FBS, 10 U/ml penicillin/streptomycin and 50 μM 2-mercaptoethanol). Primary bone marrow-derived macrophages were differentiated from marrow cells as previously described (16). Briefly, bone marrow cells were isolated from the femur and tibia of C57BL/6 mice and differentiated into macrophages using conditioned media (complete RPMI medium supplemented with 30% L929 cell culture supernatant). On the 7th day, the cells were harvested, washed, cultured in 24-well plates (1 ml/well) for 24 h in the presence or absence of WCE (1:10 ratio) or LPS (1 μg/ml) and the culture supernatant fluids were collected and stored at −80°C until used for cytokine ELISAs. Two million (2×10^6) cells/ml were used for all the *in vitro* culture experiments. In some experiments, the cells were pretreated with SB-203580 (p38 inhibitor, 10 μM), U-0126 (ERK inhibitor, 10 μM), SP-600125 (JNK inhibitor, 50 nM), Fludarabine (STAT1 inhibitor, 10 μM) or S31-201 (STAT3 inhibitor, 10 μM) for 1 h before stimulation with WCE or LPS.

Isolation of Peritoneal Macrophages

Groups of mice were inoculated with 100 μl of PBS containing 10^3 live parasites or 10^7 parasite equivalent of WCE. The mice were sacrificed at different time points (30 min to 24 h) post inoculation, and the peritoneal lavage fluid was collected as previously described (17). Macrophages from peritoneal lavage were lysed with NP40 lysis buffer and used for western blots.

Western Blot

Assessment of MAPKs, STATs, and NFKB p65 phosphorylation were determined by Western blot, as previously described (18). Briefly, ANA-1 cells, peritoneal macrophages or fully differentiated BMDMs were serum starved in petri-plates for 24 h and inoculated with WCE or LPS. At indicated times, the cells were washed with ice cold PBS, total protein was extracted using NP40 lysis buffer supplemented with protease inhibitor cocktail (1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride). The lysates (10 μg) were resolved in 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Quebec, Canada).The membranes were blocked with 5% BSA in TBST for 2 h at room temperature. The membranes were then incubated overnight with specific polyclonal rabbit antibodies against phosphorylated mouse p38, ERK1/2, JNK, STAT1, STAT3, STAT5, and NFkB p65 subunit. Thereafter, the blots were washed with TBST (5 times) and incubated with goat anti-rabbit HRP-conjugated secondary antibody and the bands were revealed by enhanced chemiluminescence (ECL) (Amersham, GEHealthcare Bioaciences, PA) reagents. Thereafter, the blots were routinely stripped and re-probed with antibodies against total p38, ERK, JNK, STAT1, STAT3 that were used as loading controls. Densitometric analysis was performed and integrated density values were presented as the ratio of phosphorylated protein over total compared with media control.

Preparation of Spleen and Liver Cells for Cytokine Analysis

At day 9 after infection, mice were sacrificed and blood were collected for serum. The spleens and livers were collected and processed into single-cell suspensions. Red blood cells in the spleen cells were routinely lysed with ACK lysis buffer, and the cells were washed with PBS and resuspended in in complete tissue culture medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, 100 U/mL Penicillin, and 100 μg/ml streptomycin). The liver samples were perfused through the right ventricle with 10 ml of ice-cold-PBS and digested with collagenase-D (125 μg/mL) for 30 min at 37°C before being homogenized. The cells were passed through a 70 μm cell strainer (VWR, Mississauga, ON, Canada) and washed by spinning at 1,200 rpm for 5 min. Washed liver cells were resuspended in 40% percoll (Sigma) and carefully layered above 70% percoll and centrifuged at 750 × g for 20 min at 22°C. The interface containing lymphocytes was collected and washed with PBS. The lymphocytes were resuspended at final concentration of 4×10^6/ml in complete tissue culture medium and cultured for 48 h. The culture supernatant fluids were collected and assayed for cytokines by ELISA.

Cytokine ELISA

The levels of cytokines (IL-1β, IL-6, IL-12p40, IFN-γ, TNF, and MCP-1) in serum of infected mice or culture supernatant fluids from BMDMs and ANA-1 cells stimulated with WCE or LPS or cultures of spleen and liver cells were determined by ELISA using paired Abs and appropriate cytokine standards (eBioscience) according to the manufacturer's suggested protocols. In another set of experiments the cells were pretreated with SB-203580 (10 μM), U-0126 (10 μM), SP-600125 (50 nM) for 1 h before

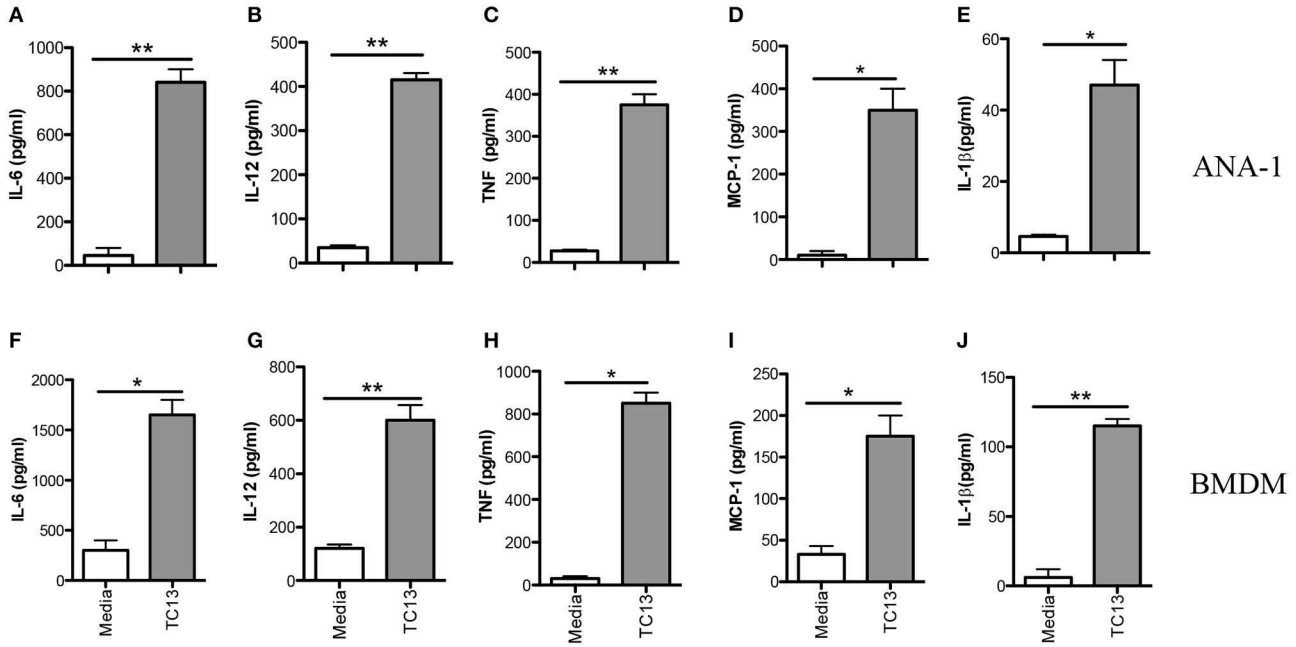

FIGURE 1 | *Trypanosoma congolense* induces cytokine production in immortalized macrophage cell lines (ANA-1) and primary bone marrow-derived macrophages (BMDMs). ANA-1 cells and BMDMs were stimulated with *Trypanosoma congolense* whole cell extract (WCE, 1:10 ratio) and after 24 h, the culture supernatant fluids were collected and assayed for cytokines by sandwich ELISA. Shown are IL-6 **(A,F)**, IL-12p40 **(B,G)**, TNF **(C,H)**, MCP-1 **(D,I)**, and IL-1β **(E,J)** levels in the culture supernatant fluids of ANA cells **(A–E)** and BMDMs **(F–J)**. The data presented are representative of three independent experiments with similar results. $^{*}p < 0.05$; $^{**}p < 0.01$.

stimulation with *Trypanosoma* whole cell extract or LPS. Supernatants were collected after 24 h and assayed for IL-6 and IL-12p40.

Statistical Analysis

Cytokine data are presented as mean ± SEM. Differences in cytokine production between groups were compared using two-tailed Student's *t*-test and two-way ANOVA. Significance was considered if $p < 0.05$. GraphPad Prism software was used to analyse the data.

RESULTS

Trypanosoma congolense (TC) Induces Proinflammatory Cytokine Production in Macrophage Cell Lines and Bone Marrow-Derived Macrophages (BMDM)

Infection of mice with African trypanosomes (including *T. congolense*) is associated with high levels of serum proinflammatory cytokines (including IL-12p40, IL-6, and TNF-α) and nitric oxide (NO) (19, 20). Indeed, death of *T. congolense*-infected mice is associated with cytokine storm leading to systemic inflammatory response like syndrome (SIRS) (8). However, the key innate immune cells and molecular mechanisms underlying recognition of *T. congolense* are not yet studied. Because macrophages play a central role in resistance to experimental African trypanosomiasis (21, 22), we investigated their role in proinflammatory cytokine production and the molecular pathways involved in this process. First, we stimulated immortalized macrophage cell line (ANA-1) with *Trypanosoma congolense* whole cell extract (WCE) for 24 h and assessed proinflammatory cytokine secretion by ELISA. WCE stimulation induced IL-6, IL-12p40, TNF-α, MCP-1, and IL-1β production in ANA-1 cells (**Figures 1A–E**). We also confirmed the induction of these cytokines in primary bone marrow-derived macrophages (BMDMs, **Figures 1F–J**). Collectively, these results show that *T. congolense* induces strong production of proinflammatory cytokines in macrophages.

T. congolense Induces MAPKs and STATs Phosphorylation in Macrophages

MAPK and STAT family of signaling molecules are important in regulating proinflammatory cytokine production in immune cells including macrophages (23). Although not yet demonstrated in *T. congolense* infection, several parasitic infections have been shown to activate MAPK and STAT signaling pathways in infected cells (24, 25). Therefore, we wished to determine whether the induction of proinflammatory cytokine production in macrophages by WCE involves activation of the MAPK and STAT pathways. We stimulated BMDMs with WCE and at different times, assessed phosphorylation of MAPK and STAT proteins by western blot. As shown in **Figures 2A–E**, WCE induced phosphorylation of ERK,

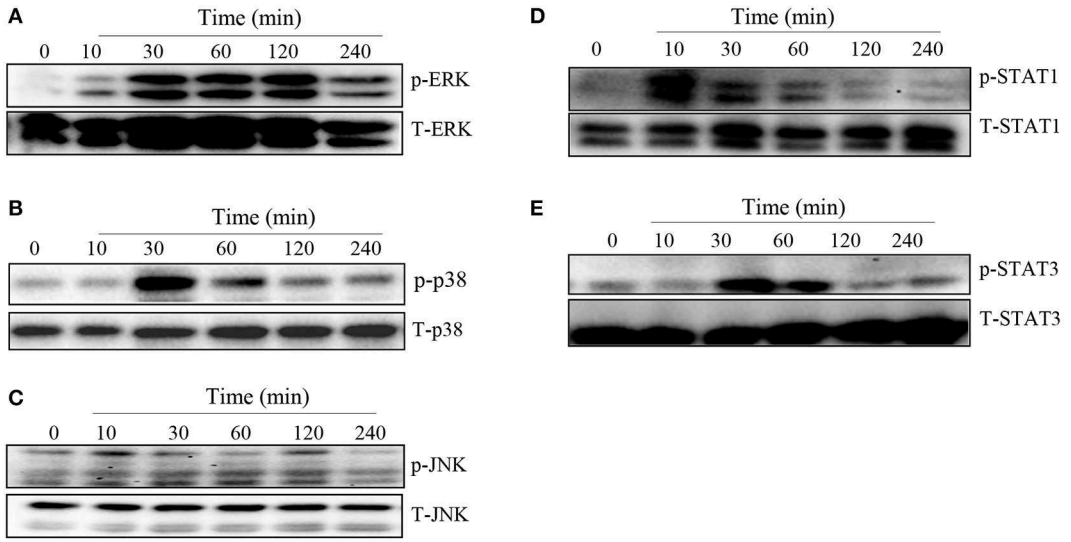

FIGURE 2 | *T. congolense* induces phosphorylation of MAPKs and STATs in macrophages. BMDMs were stimulated with WCE and at the indicated times, the cells were lysed and the lysates were assessed by western blot for phosphorylation of ERK **(A)**, p38 **(B)**, JNK **(C)**, STAT1 **(D)**, and STAT3 **(E)** proteins using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against total ERK, p38, JNK, STAT1, and STAT3 and used as loading controls. The data presented is a representative of three independent experiments with similar results.

p38, JNK, STAT1, and STAT3 proteins in primary BMDMs at various time points after stimulation. Collectively, these results suggest that *T. congolense*-induced cytokine production may be mediated through MAPK and STAT signaling pathways.

Inhibitors of MAPKs and STATs Significantly Reduce *T. congolense*-Induced Cytokine Production in Macrophages

To directly determine the involvement of MAPKs and STATs in *T. congolense*-induced cytokine production in macrophages, we performed the above experiments in the presence or absence of specific inhibitors of p38 (SB203580), p42/p44 ERK (U0126), JNK (SP600125), STAT1 (Fludarabine), and STAT3 (S31-201). Pre-treatment of BMDMs with SB203580, U0126, SP600125, and Fludarabine before stimulation with *T. congolense* WCE significantly suppressed IL-6 and IL-12p40 production by these cells (**Figures 3A,B**). Interestingly, STAT3 inhibitor, S32-201, did not affect WCE-induced IL-6 and IL-12 production, indicating that WCE-induced STAT3 signaling is not as critical as MAPKs at inducing IL-6 and IL-12 production in macrophages. As positive controls, we showed that these specific inhibitors also blocked LPS-induced production of IL-6 and IL-12p40 in macrophages (**Figures 3C,D**). Taken together, these results confirm that members of MAPK (JNK, p38, ERK) and STAT1 play important role in controlling the intracellular events that lead to the production of IL-6 and IL-12p40 in *T. congolense* treated macrophages.

T. congolense Induces MAPK and STAT Phosphorylation in Macrophages *in vivo*

Next, we evaluated whether MAPK and STAT phosphorylation induced by WCE is reproducible *in vivo*. We injected C57BL/6 mice intraperitoneally with WCE and phosphorylation of MAPKs and STATs was assessed in the peritoneal macrophages directly *ex vivo*. As shown in **Figures 4A–D**, WCE injection significantly induced phosphorylation of ERK, p38, STAT1, and STAT3 in peritoneal macrophages. In another set of experiments, we injected live parasites into C57BL/6 mice i.p. and assessed MAPK and STAT phosphorylation in macrophages from the peritoneal lavage fluid at different times after infection. As with WCE, injection of live parasites also up-regulated the phosphorylation of MAPK and STATs (**Figures 4E–H**), and leads to a concomitant increase in IL-6 and IL-12 levels in the peritoneal lavage fluids (**Figures 4I,J**). These results show that *T. congolense*-induced phosphorylation of MAPK and STATs occurs following *in vivo* infection.

MyD88 Is Involved in *T. congolense*-Induced Intracellular Signaling and Cytokine Production

The central adaptor molecule, MyD88, plays a crucial role in initiating proinflammatory cytokine production in macrophages infected with other related protozoa including *T. cruzi* (26, 27) and *T. brucei* (28). To determine whether signaling via MyD88 is crucial for *T. congolense*-induced proinflammatory cytokine production, we stimulated BMDMs from MyD88$^{-/-}$ and WT mice with WCE and performed western blot at different times to compare MAPK and STAT phosphorylation and production of proinflammatory cytokines. Deficiency of

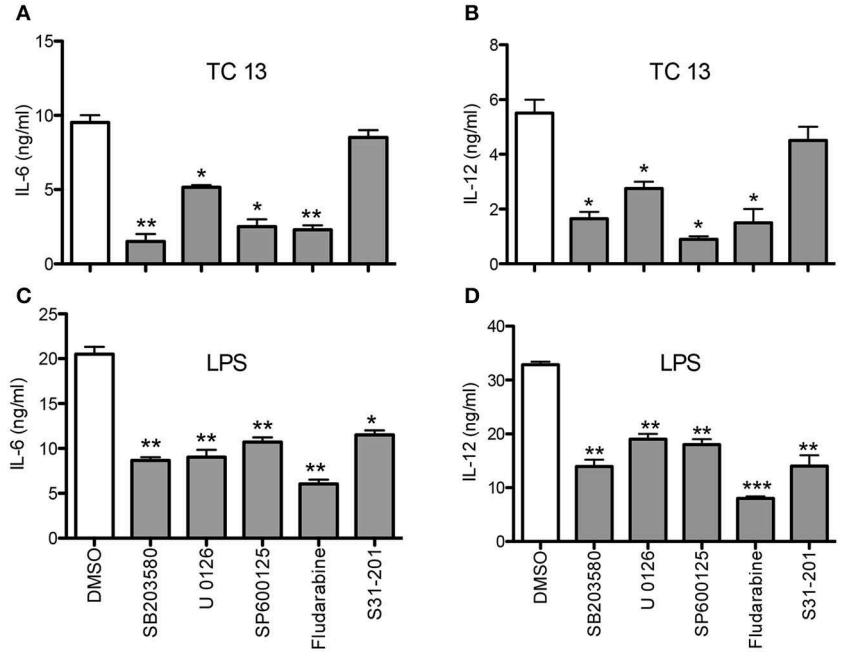

FIGURE 3 | MAPK and STAT inhibitors abrogate *T. congolense*-induced IL-6 and IL-12p40 production in macrophages. BMDMs were treated with MAPK inhibitors (U0126 for ERK, SB203580 for p38, and SP600125 for JNK) and STAT inhibitors (Fludarabine for STAT1 and S31-201 for STAT3) for 1 h prior to stimulation with WCE **(A,B)** or LPS **(C,D)**. After 24 h, the levels of IL-6 **(A,C)** and IL-12p40 **(B,D)** in the culture supernatant fluids were determined by sandwich ELISA. The data presented are representative of three independent experiments with similar results. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to cells treated with DMSO (vehicle).

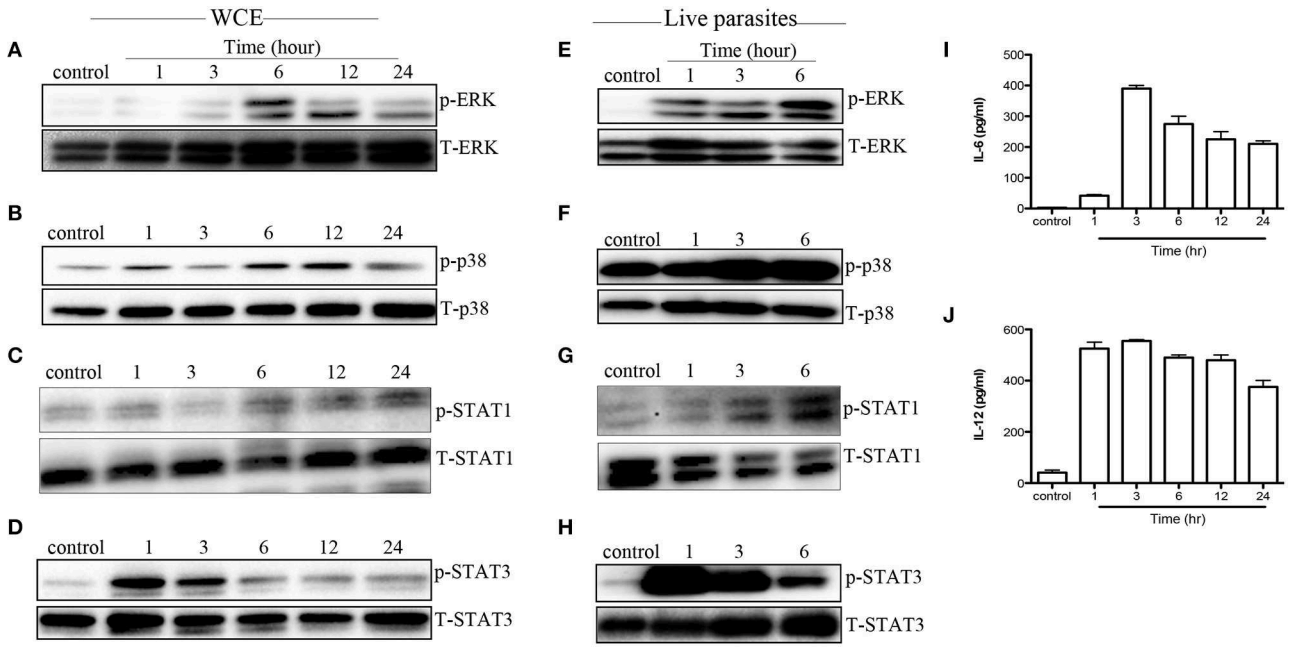

FIGURE 4 | *T. congolense* induces MAPK and STAT phosphorylation in peritoneal macrophages. C57BL/6 mice were inoculated intraperitoneally with 100 μl WCE containing 10^7 parasite equivalent **(A–D)** or 10^6 live parasites **(E–H)**. At the indicated times, mice were sacrificed and peritoneal macrophages were isolated from peritoneal wash, lysed and assessed directly for phosphorylation of ERK **(A,E)**, p38 **(B,F)**, STAT1 **(C,G)**, and STAT3 **(D,H)** by western blot. In addition, the levels of IL-6 **(I)** and IL-12p40 **(J)** in the peritoneal lavage fluid of mice inoculated with WCE were also determined by ELISA. The data presented are representative of three **(A–D,I,J)** and two **(E–H)** independent experiments with similar results ($n = 4$–5 mice per each time point).

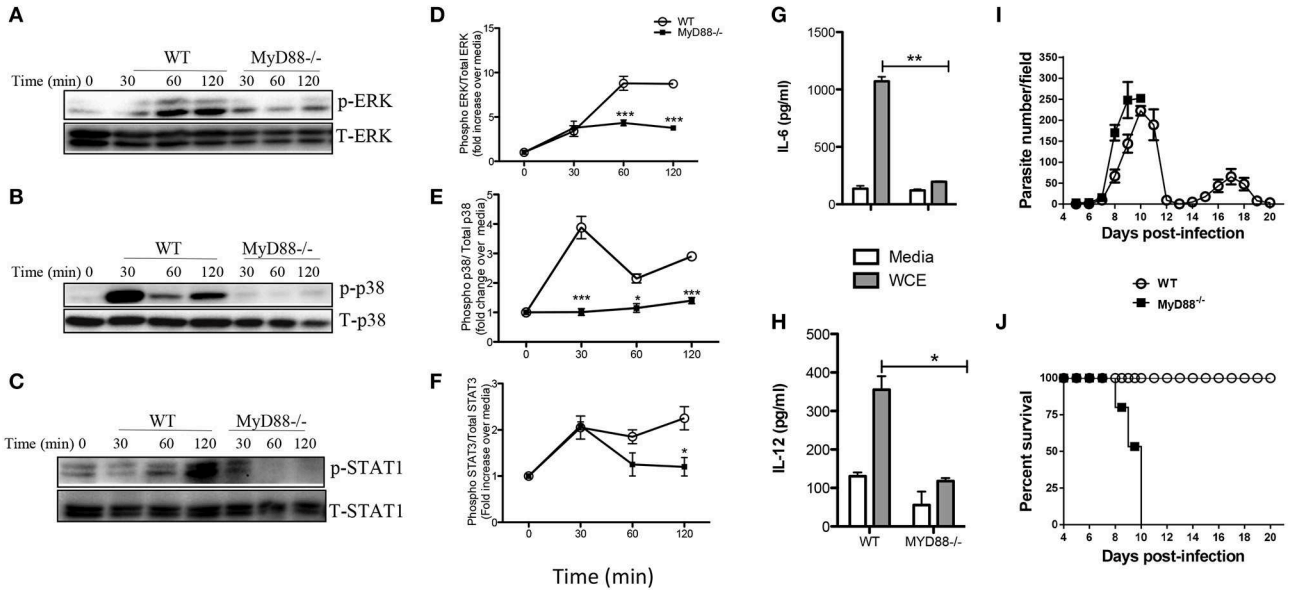

FIGURE 5 | Intact MyD88 is critical for *T. congolense*-induced MAPK and STAT phosphorylation and resistance to infection. BMDMs from WT and MyD88$^{-/-}$ mice were stimulated with WCE and at indicated times, the cells were lysed and the lysates were assessed by western blot for phosphorylation of ERK **(A,D)**, p38 **(B,E)**, and STAT1 **(C,F)** using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against total ERK, p38 and STAT1 and used as loading controls. The ratios of phosphorylated ERK **(D)**, p38 **(E)**, and STAT1 **(F)** to their respective total proteins were calculated by densitometry and plotted as line graphs **(D–F)**. In addition, the levels of IL-6 **(G)** and IL-12 **(H)** in the culture supernatant fluids were determined by ELISA. Groups ($n = 6$ per group) of MyD88$^{-/-}$ and C57BL/6 (WT) mice were infected with 10^3 *T. congolense* i.p and monitored daily for parasitemia **(I)** and survival **(J)** for about 21 days. The data presented are representative of two independent experiments with similar results. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

MyD88 completely abolished WCE-induced ERK, p38 and STAT1 phosphorylation in macrophages (**Figures 5A–F**) and this was associated with a concomitant inhibition of IL-6 and IL-12p40 production (**Figures 5G,H**).

Next, we investigated the effect of MyD88 deficiency in infection outcome by comparing parasitemia and survival period of *T. congolense*-infected WT and MyD88$^{-/-}$ mice. Infected MyD88$^{-/-}$ mice on the relatively resistant background developed higher and uncontrolled first wave parasitemia compared to WT mice and died within 10 days post-infection (**Figures 5I,J**). In contrast, all the infected WT mice controlled several waves of parasitemia and survived up to 20 days (when the experiment was terminated). The enhanced susceptibility of MyD88$^{-/-}$ mice to *T. congolense* infection was associated with significant reduction in serum levels of IL-6, TNF-α, and IFN-γ (**Figure S1**). These results show that the central adaptor molecule, MyD88, is critical for resistance to *T. congolense* infection. They further suggest the involvement of TLRs in the recognition of trypanosomal molecule and subsequent initiation of innate immune response to the parasite.

TLR-2 Is Essential for WCE-Induced MAPK and STAT Signaling and Resistance to *T. congolense* Infection Mice

Toll like receptors (TLR) are important innate immune receptors involved in recognition of conserved molecular patterns expressed by microbes and the initiation of innate immune responses. Given that activation of MyD88 is usually associated with ligation of several TLRs, we sought to determine whether TLR signaling is involved in WCE recognition by macrophages. WCE-induced phosphorylation of ERK, p38 and STAT1 and the production of IL-6, IL-12p40, and TNF-α were not different in TLR4$^{-/-}$ compared to WT macrophages (**Figures S2A–I**). In contrast, WCE-induced phosphorylation of p38, ERK, STAT1, and STAT3 was dramatically suppressed in TLR2$^{-/-}$ macrophages (**Figures 6A–H**) and this was associated with dramatic reduction in IL-6, IL-12p40, and TNF-α production (**Figures S3A–C**).

Next, we investigated whether TLR2 plays a critical role in the outcome of infection by comparing parasitemia and survival period of *T. congolense*-infected WT and TLR2$^{-/-}$ mice. Infected TLR2 deficient mice on the relatively resistant background developed higher and uncontrolled first wave parasitemia compared to WT mice and died within 10 days post-infection (**Figures 6I,J**). On the other hand, all infected WT mice controlled several parasitemia waves and survived up to 30 days that's when the experiment was terminated. The enhanced susceptibility of TLR2 deficient mice was associated with significant ($p < 0.05$) reduction in serum levels of IL-6, TNF-α, and IFN-γ (**Figures S3D–F**) and IL-6 and IFN-γ levels in their spleen and liver cell culture supernatant fluids, compared to their WT counterpart mice (**Figures 6K–N**). Interestingly, IL-10 levels in the serum (**Figure S4A**) and the production of IL-10 by spleen cells (**Figure S4B**) from infected TLR2$^{-/-}$ mice were also significantly lower than those of their WT counterpart mice,

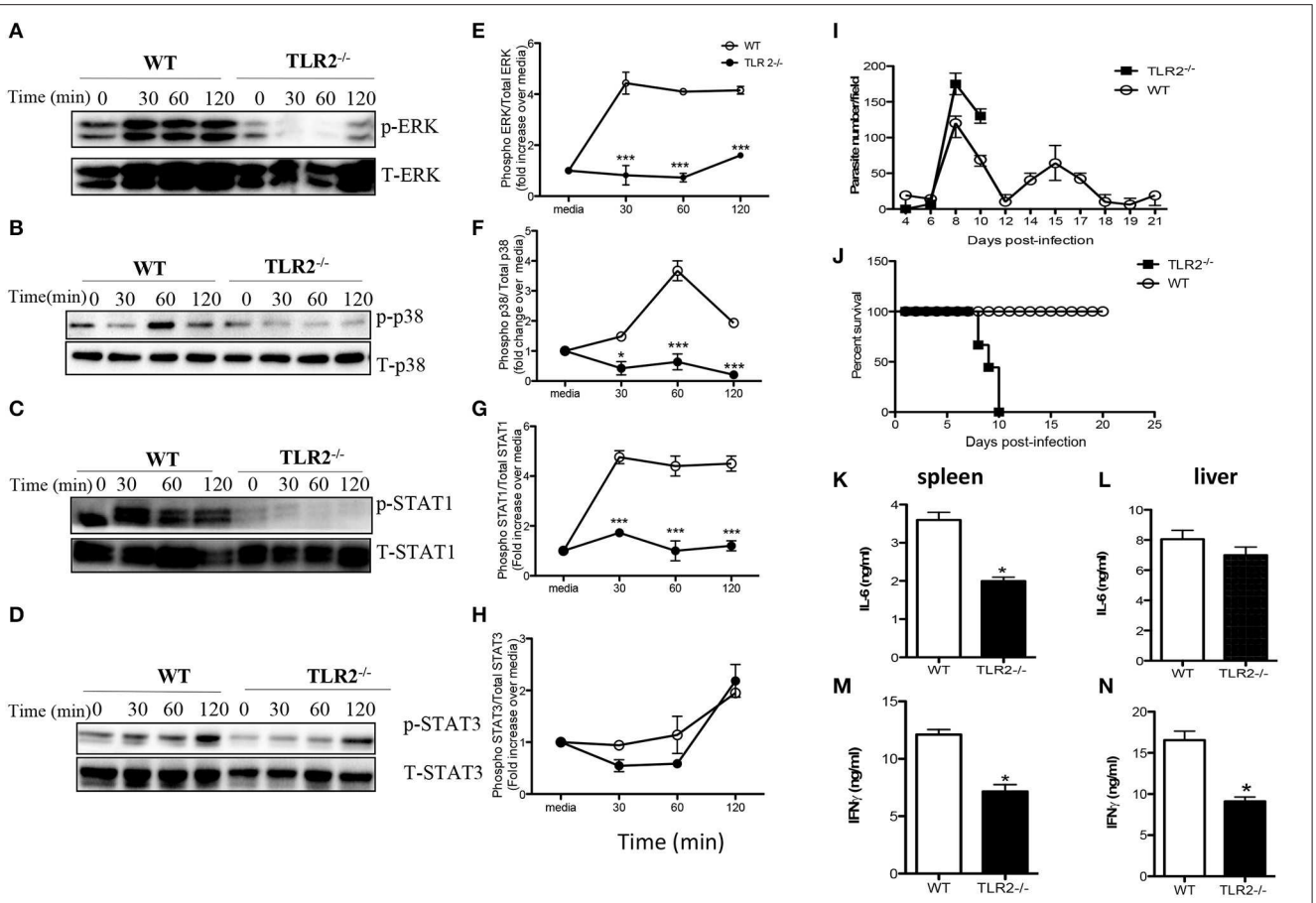

FIGURE 6 | TLR2 is essential for *T. congolense*-induced MAPK and STAT phosphorylation and resistance to *T. congolense* infection. BMDMs from WT and TLR2$^{-/-}$ mice were stimulated with WCE and at the indicated times, the cells were lysed and lysates were assessed by western blot for phosphorylation of ERK **(A)**, p38 **(B)**, STAT1 **(C)**, and STAT3 **(D)** using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against total ERK, p38, STAT1, and STAT3 and used as loading controls. The ratios of phosphorylated ERK **(E)**, p38 **(F)**, STAT1 **(G)**, and STAT3 **(H)** to their respective total proteins were calculated by densitometry and plotted as line graphs **(E–H)**. Groups ($n = 12$ per group) of TLR2$^{-/-}$ and C57BL/6 (WT) mice were infected with 10^3 *T. congolense* i.p and monitored daily for parasitemia **(I)** and survival **(J)** for about 21 days. At day 9 post-infection, some WT and TLR2$^{-/-}$ mice were sacrificed and spleen and liver cells were cultured for 48 h and the production of IL-6 **(K,L)** and IFN-γ **(M,N)** were determined by ELISA. The data presented are representative of 2–3 independent experiments with similar results. *$p < 0.05$; ***$p < 0.001$.

suggesting that the lower levels of inflammatory cytokines in the serum of these mice may not due to dampening effects of IL-10 on their production. Collectively, these results show that TLR2 is a critical innate receptor involved in the recognition of *T. congolense* and initiation of optimal protective immunity to this parasite in mice.

DISCUSSION

Acute death of mice infected with African trypanosomes is usually associated with hyper-activation of immune cells (mostly macrophages and T cells), and excessive production of proinflammatory cytokines (including IL-6, IL-12p40, IFN-γ, and TNF) leading to systemic inflammatory response like syndrome (8). However, these cytokines are also important for efficient parasite control and resistance to African trypanosomes (29) suggesting that a fine balance must be maintained for survival. We previously showed that the production of proinflammatory cytokine by splenic and liver macrophages following *T. congolense* infection contributes to disease and treatment with Berenil suppressed (but do not completely abrogate) the production of these cytokines and prevents death in the highly susceptible mice (19). The primary focus of the present study was to investigate the innate receptor and signaling mechanisms involved in *T. congolense* recognition and induction of proinflammatory cytokine production in infected mice. We showed that *T. congolense* induces proinflammatory cytokine production in both primary and immortalized macrophages. We further showed that TLR2 is the innate receptor involved in *T. congolense* recognition by macrophages and this involves the central adaptor protein, MyD88. Furthermore, we showed that activation of MAPKs (p38, ERK, and JNK) and STATS (STAT1 and STAT3) occur in macrophages following recognition of *T. congolense* resulting proinflammatory cytokine production. Blockade of MAPKs and STATs with their specific inhibitors abrogated proinflammatory

cytokine production. Collectively, these observations reveal the molecular and intracellular signaling events that lead to proinflammatory cytokine production in macrophages following their interaction with *T. congolense*.

TLRs are important for the recognition of microbes by innate immune cells (including macrophages) and initiation of innate immune response (30). Recent reports show that TLRs play an important role in initiating innate immune response against several parasitic infections including *T. brucei, T. cruzi, L. major,* and *Toxoplasma gondii* (26, 28, 31, 32). *T. cruzi*-derived glycophosphatidyl inositol (GPI) moiety that anchors the major surface glycoprotein to the cell membrane have been shown to trigger NFκβ activation and proinflammatory cytokine production in macrophages via TLR2 signaling (33). Indeed, we had previously shown that *T. congolense* induces NF-KB p65 phosphorylation in infected macrophages *in vitro* (34). In addition, DNA from *T. cruzi* stimulates cytokine production that is dependent on TLR9 and synergizes with parasite-derived GPI anchor for cytokine induction in macrophages (26). Another study showed that a subset of *T. cruzi* glycoinositolphospholipids stimulates cytokine production in macrophages via a TLR4-dependent and TLR2-independent manner (27). In contrast, *P. falciparum* GPI induces TNF production in macrophages by engaging several TLRs including TLR4, TLR2, and TLR1 (35, 36). Hitherto, no study has investigated the role of TLRs in *T. congolense*-mediated cytokine production. Our results show that the recognition of *T. congolense* leading to activation of intracellular signaling events and production of proinflammatory cytokines in macrophages is mediated through TLR2 and is independent of TLR4.

The binding of TLRs by their corresponding ligands leads to the recruitment of adaptor proteins and initiation of intracellular signaling events that ultimately result in proinflammatory cytokine gene expression. MyD88 is the most important adaptor molecule that is centrally involved in the activation of downstream signaling events following ligation of several TLRs by their respective ligands. The activation of MyD88 results in activation and/or phosphorylation of key signaling pathways including STATs and MAPKs. A recent report showed that deficiency of MyD88 leads to enhanced susceptibility to *T. brucei* infection, due in part to impaired IL-12 production by macrophages and a consequent impairment in Th1 response (28). In line with this, we found that deficiency of MyD88 leads to impaired phosphorylation of MAPKs and STATs in macrophages following *T. congolense* stimulation and this was associated with inability to control the first wave of parasitemia and death within 10 days in the relatively resistant mice. A recent report shows similar observation that the soluble variant surface glycoprotein (sVSG) of *T. brucei rhodesiense* initiates the expression of a number of proinflammatory genes including TNF, IL-12, IL-6, and iNOS by phosphorylation of ERK, p38, JNK, and NFκB pathways (37). Similarly, the GPI anchor of *T. cruzi* trypomastogotes has been shown to trigger phosphorylation of ERK and p38 in macrophages leading to NFκB activation and induction of pro-inflammatory cytokine genes (38). Furthermore, *T. gondii* has also been shown to induce MAPK and STAT3 phosphorylation in macrophages (25, 39). Thus, it appears that the activation of MAPKs and STATs may be a common pathway that is shared by protozoan parasites for the induction of inflammatory cytokines in macrophages.

TLRs recruit several TIR domain-containing adaptors, such as MyD88, TRIF, TIRAP/MAL, and TRAM for the induction of pro-inflammatory cytokine genes (40). MyD88 is recruited by extracellular TLRs, such as TLR2 and TLR4 and also associates with endosomal TLRs by binding to different lipids. However, TLR4-dependent activation of inflammatory genes occurs through MyD88 dependent and independent pathways (40–42). The MyD88-independent TLR4 signals occur through TRIF, an adaptor molecule that interacts with TRAF3 and TRF6 resulting in NF-κB and MAPKs activation and induction of several pro-inflammatory cytokines (40). Although not tested here, it is possible that TIRAP could indirectly contribute to proinflammatory cytokine gene activation and hence play a role in the overall immunity to *T. congolense* infection given that this adaptor molecule contributes to TLR2 signaling.

Although our *in vitro* and *in vivo* studies showed that TLR2 is critical for *T. congolense*-induced production of proinflammatory cytokines in macrophages, we found that deficiency of TLR2 (as seen in TLR2$^{-/-}$ mice) results in increased susceptibility to the infection. TLR2$^{-/-}$ mice on the usually resistant background were unable to control their first wave of parasitemia and succumbed acutely to the infection within 8–10 days post-infection. This finding suggests that TLR2 dependent immune activation plays a critical role in the overall immunity to *T. congolense* infection in mice. Thus, in macrophages (and perhaps other innate immune cells), TLR2 dependent recognition of *T. congolense* triggers cytokine responses that may be critical for initiating protective adaptive immunity necessary for effective parasitemia control. In line with this, we found that serum levels of IL-6, TNF-α, and IFN-γ in infected TLR2$^{-/-}$ mice were lower than those in infected WT mice.

Although our study clearly showed the importance of TLR2 in proinflammatory cytokine production and resistance to *T. congolense* infection, the parasite molecule that is recognized by this innate immune receptor remains to be defined. Recent studies have shown that recognition of *T. cruzi* and *P. falciparum* GPI molecules by TLR2 on macrophages leads to activation and production of proinflammatory cytokines (26, 43, 44). Given that *T. congolense* variant surface glycoprotein is attached to the cell membrane via a GPI anchor, it is conceivable that *T. congolense* GPI-derived molecule may be the critical ligand for TLR2 in our system. In line with this, we observed that *T. congolense*-induced MAPK and STAT phosphorylation and production of proinflammatory cytokines were unaffected in macrophages from TLR4 deficient mice, suggesting that TLR4 signaling is not important for cytokine production in macrophages in this model. However, it is likely that the induction of proinflammatory cytokine production and activation of innate immune response following *T. congolense* infection may involve interactions between several TLRs that signal via MyD88. Further studies are required to clearly delineate the key parasite molecules involved in TLR2-MyD88 dependent resistance to *T. congolense* infection.

In conclusion, our studies identify for the first time TLR2- and MyD88-dependent activation of MAPKs and STATs as key intracellular events that are involved in cytokine production and enhanced resistance *T. congolense* infection in mice. Deficiency of TLR2 leads to uncontrolled first wave of parasitemia and acute death in an otherwise relatively resistance C57BL/6 mice. Understanding the receptors, adaptor proteins and the complex signaling pathways involved in immunity to African trypanosomiasis is of great interest and could eventually be used to develop novel strategies to enhance protective immunity and prophylaxis against the infection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Manitoba Animal Care Committee and experiments were carried out in accordance with the regulation of the Canadian Council on Animal Care.

AUTHOR CONTRIBUTIONS

SK, CO, RS, FO-A, PJ, and JU: design, acquisition, analysis, and interpretation of data. SK and CO: drafting. SK, CO, and JU: critical review. All authors reviewed and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02673/full#supplementary-material

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Plasmodium falciparum Merozoite Associated Armadillo Protein (PfMAAP) Is Apically Localized in Free Merozoites and Antibodies Are Associated With Reduced Risk of Malaria

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Understanding the functional role of proteins expressed by *Plasmodium falciparum* is an important step toward unlocking potential targets for the development of therapeutic or diagnostic interventions. The armadillo (ARM) repeat protein superfamily is associated with varied functions across the eukaryotes. Therefore, it is important to understand the role of members of this protein family in *Plasmodium* biology. The *Plasmodium falciparum* armadillo repeats only (*Pf*ARO; Pf3D7_0414900) and *P. falciparum* merozoite organizing proteins (*Pf*MOP; Pf3D7_0917000) are armadillo-repeat containing proteins previously characterized in *P. falciparum*. Here, we describe the characterization of another ARM repeat-containing protein in *P. falciparum*, which we have named the *P. falciparum* Merozoites-Associated Armadillo repeats protein (PfMAAP). Antibodies raised to three different synthetic peptides of PfMAAP show apical staining of free merozoites and those within the mature infected schizont. We also demonstrate that the antibodies raised to the PfMAAP peptides inhibited invasion of erythrocytes by merozoites from different parasite isolates. In addition, naturally acquired human antibodies to the N- and C- termini of PfMAAP are associated with a reduced risk of malaria in a prospective cohort analysis.

Keywords: Malaria, armadillo, invasion, merozoites, antigen, antibodies, recombinant protein

INTRODUCTION

Human malaria is caused by several species of the genus *Plasmodium*, with the majority of deaths attributed to *Plasmodium falciparum*. The life cycle of the parasite is multifaceted involving both the mosquito vector and the human host, with asexual multiplication of the parasite in the blood responsible for the clinical manifestations of the disease. Asexual replication requires successful

invasion of the erythrocyte by the merozoite stage. This process is complex, involving proteins released from the apical organelles or located on the merozoite surface (1, 2).

Proteins stored in organelles such as the micronemes, rhoptries, and dense granules have been extensively studied to define their roles in merozoite invasion (1, 2). Notable invasion-linked protein families include the *P. falciparum* reticulocyte binding like protein homologs (PfRH1, 2a, 2b, 4, and 5) (3–8), the erythrocyte binding antigens (PfEBAs; EBA 140, 175, 181 & EBL1) (9–14) and the rhoptry neck proteins (RONs) (15, 16). Other proteins such as AMA1 (17) have been shown to play critical roles during the process of merozoite invasion.

Our interest in the PF3D7_1035900 protein arose from a survey of the antigen-rich chromosome 10 cluster. A region that contains a number of well-characterized putative vaccine candidates including, the MSP3/6 protein family (18, 19), GLURP (20, 21), liver stage antigen 1 (22), to name but a few. In addition to its location, transcriptional data demonstrated a peak of expression at the late schizont/early merozoite stage (23, 24), a profile that hinted at a biological importance for the late schizont and merozoite stages of the parasite.

To understand the role this gene played in parasite biology our initial investigations determined that PF3D7_1035900 was a member of the armadillo protein family, a family with pleiotropic functions that warranted further investigation as a potential intervention target. The eukaryotic armadillo repeat proteins are involved in diverse roles including cell adhesion, cell motility, cytoskeletal arrangement, molecular chaperones, cell signaling/sensing, and nuclear import (25, 26). In apicomplexan parasites, the armadillo repeat containing proteins are being characterized for their role during parasite development. The importance of this protein family is highlighted by their involvement in fundamental processes essential to parasite biology, including but not restricted to gene regulation and cytokinesis. Essential processes have been linked with the previously described *P. falciparum* ARM Repeats Only (PfARO) and *P. falciparum* Merozoite Organizing Proteins (PfMOP) (22), respectively. The putative function of PfARO has been assigned through studies using the *Toxoplasma gondii* paralog, TgARO (20, 21). In this study, we describe the characterization of another member of the armadillo repeat family of proteins, encoded by gene locus PF3D7_1035900, which lies in an antigenic rich region of chromosome 10 among members of the *msp3* gene family and several other antigen genes.

The gene shows peak expression late in the developmental cycle in the schizont. Antibodies raised to synthetic peptides demonstrate staining of the apical tip in free merozoites and those within the schizont. We propose the name *Plasmodium falciparum* merozoite associated armadillo protein (PfMAAP) due to its association with fully segmented merozoites within the mature schizont and with free merozoites. Furthermore, we show that the recombinant proteins based on the N-, central repeat and C- terminal regions are recognized by antibodies in plasma of malaria exposed individuals, with antibodies to the N and C- terminal conserved domains being associated with a lower prospective risk of contracting malaria.

RESULTS

PfMAAP Is an Armadillo (ARM)-Repeat Containing Protein

To determine the putative function of the PfMAAP protein (PF3D7_1035900) we interrogated the amino acid sequence to gain insight into the protein product. Aside from a signal peptide (amino acid 1 to 21; **Figure 1A**), we identified an armadillo (ARM)-repeat domain comprised of 5 repeats (aa 144-504; **Figures 1A,B**) and an overlapping Pumilio homolog domain (aa 202–566; **Figure 1A**). We analyzed the amino acid sequences for 16 isolates using the REPeats and their PERiodicities (REPPER) server (https://toolkit.tuebingen.mpg.de/#/tools/repper), which identifies short gapless repeats in both protein and nucleotide sequences (27). Both the laboratory and field isolates showed that the central repeat region always started at amino acid position 144 but varied in length from 166 (7G8 isolate) to 467 (GB4 isolate) amino acids (**Figure S1** and **Table S1**). Amino acid sequence alignments also showed high levels of sequence conservation within the *P. falciparum* isolates (**Figure S1**) and between *P. falciparum* (3D7) and available sequences for non-human primate malarias at both the N- and C-terminal regions (**Figure S2**). The position of the repeat regions in all isolates was validated using the REPPER server (27, 28) (**Figure S3** and **Tables S1, S2**). Further investigation of the PfMAAP protein was performed by *in silico* structural modeling using the I-Tasser structural prediction server (29–31). This was done to determine the putative structure of the proteins and to identify structural, and potentially functional, homologs of the PfMAAP protein. Using the I-Tasser structural prediction algorithm, the resolved crystal structures for three armadillo-repeat containing proteins, showing close structural homology with the PfMAAP protein were identified (**Figure 1B**). These include, β-catenin, a molecule shown to be involved in cadherin-based adhesion and implicated in cerebral malaria (27, 29, 30); the symmetrical sisters (SYS)-1 protein, functionally similar to β-catenin (32); and Plakophilin 1, also functionally similar to β-catenin **Figure 1B**). All of these which provides additional evidence of as to the potential biological significance of the PfMAAP protein.

The PfMAAP region was also identified in the available *Plasmodium* sequences from 6 *Laverania* species infections of primates (**Figure S2**), suggesting an ancient origin for the protein family. Using the 3D7 isolate as the reference sequence, we show that the signal peptide and an additional 100 amino acids (position 22–121) have high levels of sequence identity between the human (3D7) and *Laverania* primate species at the N- and C-terminal regions (**Figure S2**). However, the repeat regions varied extensively in the composition of the repeats, ranging from 190 to 402 aa in length (**Figure S2** and **Table S2**).

PfMAAP Is Expressed Late in Erythrocytic Stage Development

Transcript expression analysis of three *P. falciparum* laboratory-adapted strains (3D7, W2mef, Dd2) were performed across the asexual blood stage cycle at 8-h intervals. The expression levels were evaluated in triplicate and were the results of two separate

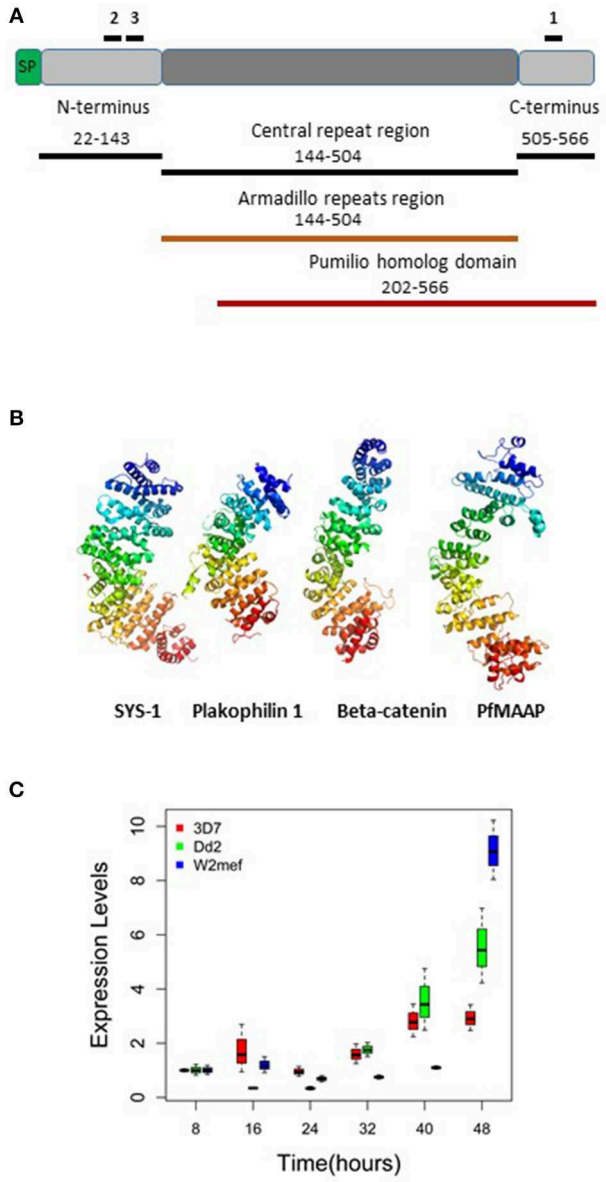

FIGURE 1 | Summary of *Pf*MAAP protein characteristics, including location of peptides and recombinants and transcript expression levels. **(A)** Cartoon of the *Pf*MAAP protein highlighting the location of the *Pf*MAAP peptides, shown with black lines above the scheme and labeled 1, 2, and 3. The position of the *Escherichia coli* expressed conserved N- and C-termini and central repeat region are shown below the scheme, with black lines and corresponding amino acid positions. The Armadillo repeat region (orange, amino acid position corresponds to central repeat region) and the Pumilio homolog domain (red line, with amino acid positions shown). **(B)** The predicted structural model for *Pf*MAAP is shown as a ribbon diagram preceded by other known armadillo repeat containing proteins, Sys-1 (symmetrical sisters-1, 3C2H in Protein Data Bank), Plakophilin-1 (1XM9) and B-catenin (1JDH), highlighting the structural similarity between *Pf*MAAP and other armadillo repeat containing proteins. **(C)** Boxplots showing differential expression of *Pf*MAAP across the asexual stage of development for four laboratory isolates and one clinical isolate. Transcript fold change is plotted against time (hours post invasion), with peak expression at 40 or 48 h post merozoites invasion. Transcript experiments were conducted in triplicate and conducted on two independent occasions.

experiments. The transcript expression profile demonstrated that expression of PfMAAP peaked in the later stages of parasite development at around 40–48 h post invasion (**Figure 1C**). This finding was supported by transcript data previously reported on Plasmodb (https://plasmodb.org/plasmo/) and in (24, 32).

PfMAAP Is Expressed in Both Merozoites and Schizonts

Three peptides spanning 14 amino acids, located at the C- (PfMAAP1) and N-terminal (PfMAAP2 and 3) regions (**Figure 1A**) were used to immunize three rabbits per group. The polyclonal sera obtained was then used to identify the location of the expressed gene product by immunofluorescence assay and thereby validate the presence of native epitopes within the peptides by recognition of the native parasite protein. Antibodies raised to all three peptides showed localization of the native protein by immunofluorescence assay (IFA) on free merozoites and those located within fixed preparations of mature schizonts (**Figures 2A–C**). All three antibody preparations showed similar staining patterns within mature schizonts and free merozoites (**Figures 2A–C** and **Figure S4**). As a result, all subsequent IFA experiments will simply be referred to as α-PfMAAP antibody. The staining patterns observed showed a clear merozoite surface staining pattern in developing schizonts and a predominantly apical staining in free merozoites and on those within mature rupturing schizonts (**Figure 2A**). The staining pattern was compared by co-localization with relative to PfAMA1 (micronemes), PfRAP2 (rhoptry bulb), and PfRON4 (rhoptry neck) (**Figures 2A–C**). Co-localization with α-AMA1 antibodies (a micronemal marker), showed apical staining of merozoites within the mature schizont (**Figure 2A**) and on free merozoites (**Figure 2B**), with a small proportion showing diffuse surface staining of the merozoite by the α-PfMAAP antibody (**Figure 2A**, top panel). Punctate apical staining was also observed with co-localization of the α-PfMAAP with the α-PfRAP2 (a marker for the rhoptry bulb) and α-PfRON4 (a rhoptry neck marker) (**Figures 2A,B**); although in the latter two cases, no additional peri-merozoite staining was observed. To clarify the merozoite surface-like staining observed with the α-AMA1 antibody, an additional co-localization assay was performed with an α-MSP1 antibody, as a marker for the merozoite surface. The results confirmed the merozoites surface-like staining of merozoites within the mature intact schizonts. To further evaluate the distribution of the different staining patterns, a total of 200 intact schizonts were assessed for the staining pattern; 99% ($n = 198$) showed the MSP1-like staining with 1% showing a diffuse staining pattern. The apically concentrated staining pattern observed in released merozoites was observed in 99.5% of the evaluated merozoites ($n = 995$) with the remaining 0.5% ($n = 5$) showing a diffused staining pattern (summarized in **Figure 2D**). In addition, we examined purified schizont extracts from three laboratory isolates (3D7, W2mef, and Dd2) by Western blot analysis to determine the relative expression of PfMAAP protein in each isolate. Screening of the blots with the α-PfMAAP antibody demonstrated that the protein

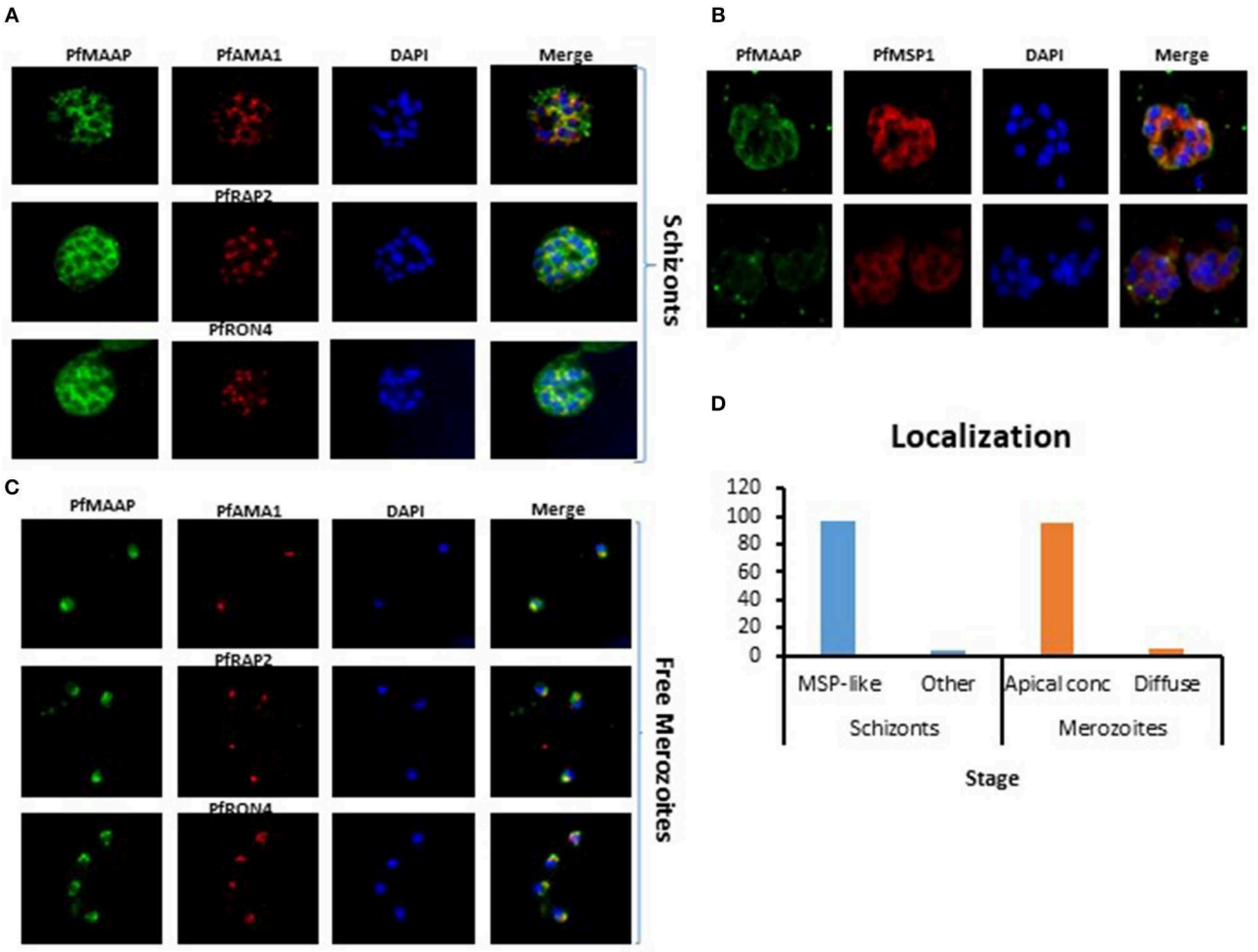

FIGURE 2 | Localization of *Pf*MAAP in schizonts and merozoites. **(A)** Co-localization of anti-*Pf*MAAP peptide antibody reactivities (green) with the apical markers *Pf*AMA1 (microneme, red), *Pf*RAP2 (rhoptry bulb, red) *Pf*RON4 (rhoptry neck, red). Staining shows clear punctate apical staining for all three apical markers; **(B)** anti-*Pf*MAAP (red) co-localization with inner membrane complex (IMC) protein GAP45 and the merozoite surface markers *Pf*MSP1 (green). **(C)** The localization of anti-*Pf*MAAP antibody reactivities (green) in free merozoites relative to *Pf*AMA1, *Pf*RAP2, and *Pf*RON4. **(D)** Percentage (%) representation of the different staining patterns observed in the schizont stage ($n = 500$) as either MSP-like or any other pattern and in merozoites ($n = 500$) as either punctate apical localization or diffuse was performed using FIJI Image J. DAPI staining of the nuclei is shown in blue and the images are shown in the final column (merge). Fifty images were taken per antibody tested using an Olympus model BX41 fluorescent microscope with a x100 oil-immersion objective.

was expressed in each of the three isolates tested (**Figure 3**), which was in keeping with transcriptomic analysis reported in (23, 24) and on Plasmodb (https://plasmodb.org/plasmo/).

Anti-PfMAAP Antibodies Inhibit Merozoite Invasion of Erythrocytes

To assess the potential functional importance of antibodies raised to the N- (PfMAAP2 and 3) and C-terminal (PfMAAP1) domain peptides (**Figure 1A**), each antibody was assessed in a growth inhibition assay (GIA). Purified schizonts from three laboratory isolates (3D7, Dd2, and W2mef) were cultured with fresh red blood cells in the presence of the antigen-specific antibodies or the equivalent amounts of purified antibodies from pre-immune sera, in a dose dependent manner (0, 100, 250, and 500 μg/ml). Both α-PfMAAP1 (C-terminal) and α-PfMAAP2 (N-terminal) inhibited parasite invasion of the Dd2 parasite strain by 15 and 25%, respectively at 0.5 mg/ml antibody concentration, whereas α-PfMAAP3 (N-terminal) inhibited parasite invasion of red blood cells by >60% at 0.5 mg/ml (**Figure 4A**). Similar levels of invasion inhibition were achieved for 3D7 (**Figure 4B**) and W2mef (**Figure 4C**), with the antibodies showing a dose dependent merozoites invasion inhibition.

Antibodies Against PfMAAP Are Associated With Reduced Risk of Clinical Malaria

To investigate if PfMAAP might be a target of naturally acquired immunity, we expressed three recombinant proteins, based on the N- and C-terminal regions and the central polymorphic

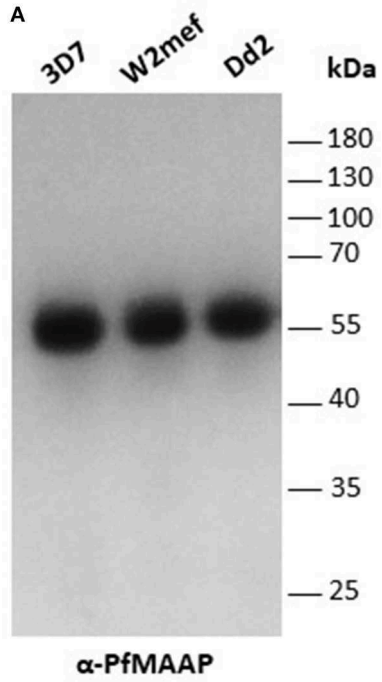

FIGURE 3 | *Pf*MAAP is expressed in multiple *P. falciparum* strains. **(A)** *Pf*MAAP1 detection of the protein in Western blot from 3D7, W2mef, and Dd2 schizont extracts. Molecular weight (kDa) indicated by the side. Molecular weight (kDa) indicated by the side.

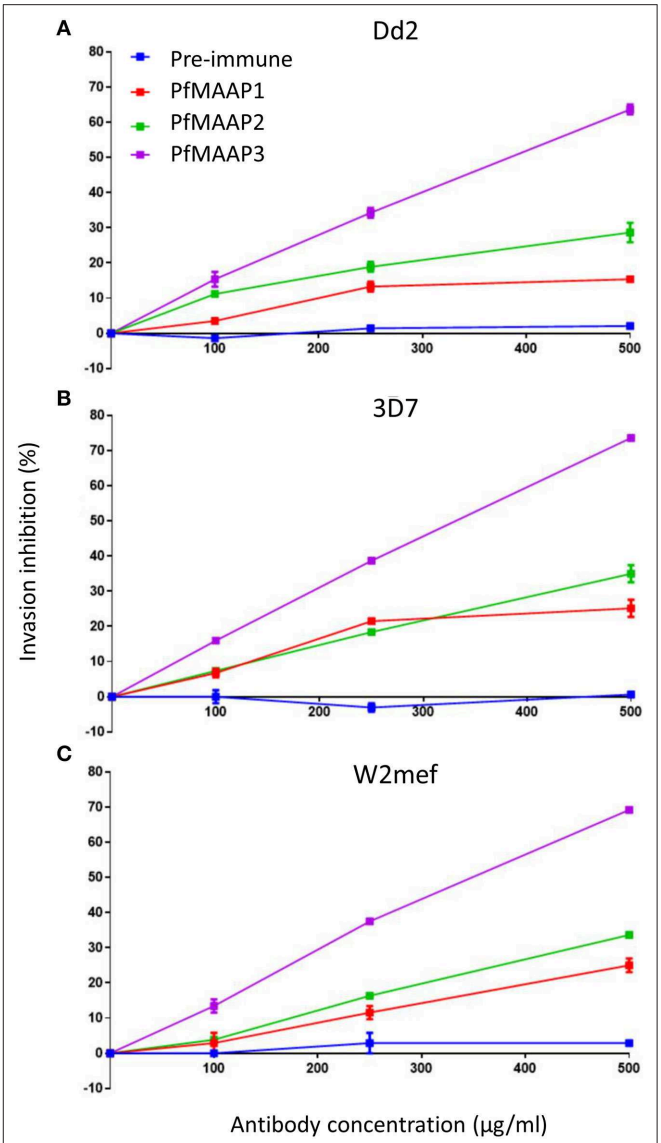

FIGURE 4 | *Pf*MAAP antibodies inhibit merozoites invasion. Evaluation of the *Pf*MAAP polyclonal antibodies in a dose-dependent manner for **(A)** Dd2 **(B)** 3D7 and **(C)** W2mef parasite isolates. The antibody reactivity for each is shown, *Pf*MAAP1 (red line), 2 (green line) and 3 (purple line), with the pre-immune negative control shown in blue. Percentage (%) invasion inhibition is plotted on the y-axis with the antibody concentrations (µg/ml) plotted on the x-axis. Pre-immunization antibodies were used as the negative control. All the assays were conducted in triplicate as two separate experiments with the plots displayed as n ± sem.

repeat region of PfMAAP (**Figure 1A**). Each protein was expressed in *E. coli* as soluble GST-tagged fusion proteins and resolved on SDS-PAGE (**Figure 5A**). ELISA was performed on plasma samples collected in Chonyi village (33) to measure IgG reactivity against each antigen fragment. The antibody responses to each antigen fragment increased with age (**Figure 5B**). Interestingly, the highest responses were seen to the central repeat region (**Figure 5B**), which corresponds to the armadillo repeat region within PfMAAP. There was a reduction in the prospective risk of clinical malaria in the subsequent 6 months after plasma collection in Chonyi associated with antibodies to both the N- and C-terminal antigens (**Table 1**). Although this association was not seen to the central repeat armadillo region (**Table 1**).

DISCUSSION

A critical step toward understanding the processes underlying parasite development, evasion of the immune system and the mechanisms involved in the selection and invasion of host cells, is understanding the role each protein plays in parasite biology. It is only through this that novel drug targets, diagnostics, or putative vaccine targets can be identified, and their role and relative importance be understood.

The armadillo super-repeat protein family appears to be involved in a variety of fundamental processes including cytoskeletal organization, cell-cell adhesion, organelle biogenesis, and signaling. Despite the variety of functions associated with this protein family across a number of diverse species, the key aspect they share is the presence of the tandemly arranged armadillo repeats. Investigations into the role(s) of the armadillo repeat family of proteins play in the apicomplexa is still in its infancy but there is evidence about some of the essential roles this family of proteins play during parasite development (34). With their previously derived function in eukaryotic cells (26), identifying any additional family members in *Plasmodium* spp.

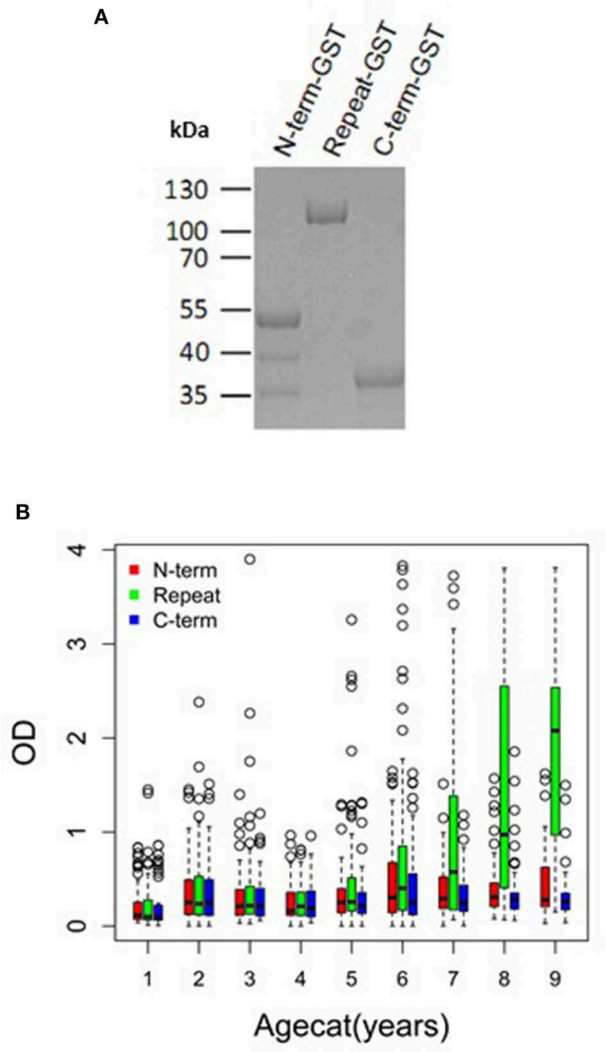

FIGURE 5 | *Pf*MAAP antibodies are associated with naturally acquired immunity. **(A)** SDS PAGE showing the purified expressed and purified GST-tagged *Pf*MAAP protein regions; N-term-GST, Central Repeat-GST, and C-term-GST. Antibody responses (OD) across all ages (October 2000) to the truncated *Pf*MAAP recombinant antigens in **(B)** Chonyi ($n = 518$). Age categories are as follows: 1 = 1–2 year, $n = 54$; 2 = 3–4 year, $n = 55$; 3 = 5–6 year, $n = 55$; 4 = 7–8 year, $n = 59$; 5 = 9–10 year, $n = 59$; 6 = 11–15, $n = 96$; 7 = 16–30 $n = 57$; 8 = 31–50, $n = 56$; and 9 = 51–82, $n = 27$.

TABLE 1 | Association between the presence of serum IgG to the panel of 3 antigens in children aged <11 years and parasite slide positive in October 2000 in the Chonyi village, and the occurrence of malaria over the following 6 months.

Antigen	[a]Proportion of children acquiring malaria who were: IgG positive	IgG negative	Univariate IRR (95% CI)	[b]Multivariate IRR (95% CI)	*P*
[c]Chonyi village (<11 years and parasite slide positive $n = 119$)					
N-term	18% (7/39)	41% (33/80)	0.44 (0.21–0.90)	0.47 (0.25–0.89)	**0.021***
Repeat	20% (10/49)	43% (30/70)	0.48 (0.26–0.88)	0.58 (0.31–1.04)	0.068
C-term	20% (8/40)	41% (32/79)	0.49 (0.25–0.97)	0.52 (0.28–0.93)	**0.028***

CI, Confidence Interval; IRR, Incidence Risk Ratio.
[a]Number of individuals developing malaria/the total number of individuals that were IgG positive or negative.
[b]The incidence Risk Ratio was estimated from multivariate analysis after adjusting for age and reactivity to Plasmodium falciparum schizont extract in a generalized linear models.
[c]Analysis focused on individuals who were parasitaemic at the time of serum sampling in October 2000.
**P <0.05.*

may illuminate different aspect of parasite biology with regards to interventions, potentially in the form of drugs, or vaccines.

To date, two armadillo repeat proteins have been described in *Plasmodium falciparum*. The *P. falciparum* armadillo-repeat only (*Pf*ARO), which bears similarity to β-catenin, an important cell-to-cell signaling molecule found in animals including humans (35). In *P. falciparum* β-catenin appears to be involved in both nuclear and rhoptry biogenesis (36). The homologous protein in *Toxoplasma gondii* (*Tg*ARO) is a multifunctional protein with similar functions to *Pf*ARO, such as rhoptry positioning and biogenesis. Importantly, *Tg*ARO can be functionally complimented by the orthologous *Pf*ARO (37–39). The *P. falciparum* merozoite organizing protein (*Pf*MOP), shown to localize to the inner membrane complex (IMC) and apical tip of the invasive merozoite stage of *P. falciparum*, appears to have a role in the biogenesis of the IMC as well as in rhoptry positioning (34, 36).

In this study we show the characterization of the *Pf*MAAP protein, initially classified as the hypothetical protein, M566. The protein was also briefly assigned to the MSP3 family, but lacked the C-terminal domain that is a defining characteristic of the family (40). We demonstrate that the protein product is expressed around 40 h post invasion, which is supported by previously published microarray proteomic and transcriptomic data (24). In keeping with the *T. gondii Tg*ARO and the *P. falciparum Pf*ARO and *Pf*MOP our results also show a clear association of *Pf*MAAP with the mature schizont and free merozoites, particularly with the apical tip.

We also demonstrate a dual localization pattern, of a punctate apical staining profile and some merozoite surface staining. The dual localization pattern observed may be associated with the myriad of functions associated with this protein family and may also be due to the early release of the *Pf*MAAP protein from the apical organelles. *Pf*MAAP has a signal peptide but lacks a transmembrane domain or a GPI-anchor and based on existing literature and sequence interrogation of the proteins there appears to be no obvious PEXEL/HT motif (41, 42) or any features indicative of a PEXEL/HT negative exported protein (PNEP) (43). Gene deletion studies of the *Pf*MAAP gene results in a reduction in growth (44) suggesting either a non-essential role in parasite development, functional redundancy or one that has a more pronounced effect elsewhere in the lifecycle.

Amino acid alignments of *P. falciparum* with available sequences for 6 non-human primate species (*Laverania* subgenus

of *Plasmodium*), including *P. praefalciparum* G01 and *P. reichenowi* CDC and G01, show high levels of sequence identity at both the N- and C-termini, including the signal peptide. This level of conservation suggests a possible evolutionarily conserved function for the PfMAAP protein that has yet to be defined.

Unlike the other Armadillo repeat containing genes described in *P. falciparum*, *Pf* MAAP showed significant invasion inhibitory activity. Most importantly there was a statistically supported association between having antibody responses to the N- and C-terminal regions of PfMAAP and protection from malaria in Chonyi (22–53 infectious bites per year), although this association was not reflected in responses to the central repeat region. This difference in antibody responses to different parts of the same protein reflects what has been described for vaccine candidates and well-characterized markers of seroincidence in the same study sites, including MSP1 block 2, MSP1-19 and MSP2 (45, 46). The association between the conserved N- and C-terminal regions of *Pf* MAAP with *in vitro* invasion inhibition, together with the cohort study analysis strongly suggests that this protein warrants further investigation as a potential target of naturally acquired immunity. Thus, understanding the role the PfMAAP protein plays in parasite biology may yield important targets for intervention strategies.

MATERIALS AND METHODS

Ethics Statement

Ethical approval for the use of the serum samples for use in this study had previously been obtained from the Kenyan National Ethics Committee, the University of Oxford, and the London School of Hygiene and Tropical Medicine as detailed in (47). Ethical approval for the Kenyan study on samples from human subjects was obtained from the Kenya National Research Ethics Committee, the University of Oxford, and the London School of Hygiene and Tropical Medicine. Written informed consent was obtained from a parent or guardian of each child contributing a blood sample and also from participating adults (47). Rabbit antibodies were obtained commercially by immunization under a commercial subcontract (Genscript). All animal work protocols were performed under the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accreditation, following guidance written by the National Research Council of the U.S. National Academy of Sciences; and the Office for Animal Welfare (OLAW) certification, demonstrating an international commitment to responsible animal care and use.

Parasite Cultures

Plasmodium falciparum laboratory isolates Dd2, W2mef, and 3D7 were cultured in complete RPMI-1640 (Sigma) (supplemented with 0.5% Albumax II (Gibco), 20 mg hypoxanthine, 2 g sodium bicarbonate (Sigma) and 0.05 mg/ml gentamicin sulfate (Sigma) using human group O^+ erythrocytes at 4% hematocrit in a mixed gas environment (93% nitrogen, 5% CO_2, and 2% oxygen; Air Liquide, Birmingham, United Kingdom) at 37 °C. Merozoites were purified after allowing schizonts to burst in the absence of fresh erythrocytes and pelleted at 4,000 rpm for 10 min. Parasite synchronizations were performed by treating mixed stages cultures with 5% D-Sorbitol (Sigma). Ring stages were then allowed to grow to schizonts. For tighter synchronizations, Percoll purified schizonts were allowed to invade over a 2-h period followed by Sorbitol treatment as describe above. For the time points, samples were collected every 8 h over a single cycle and stored in Trizol at −80°C freezer. RNA was extracted from each sample for subsequent gene expression analysis.

Homology Modeling

A 3D predicted structural model of the full-length PfMAAP was obtained following submission to the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (29–31). The most robust model was further analyzed and edited using the PyMOL 2.2 software. Comparative analysis with known armadillo repeats proteins was performed using models submitted to the Protein Data Bank (PBD at https://www.rcsb.org/).

Peptide Synthesis, Recombinant Proteins, and Polyclonal Antibody Generation

Three peptides (1-CQGEKVNKNDLNDAS, 2-FTENKEQKNEEVPMC, 3-VVNDGEEVKTEYVSC) were synthesized from the 3D7 amino acid template (**Figure 1A**) based on antigenicity, surface exposure and hydrophilicity scores (Genscript, US). Two of the peptides were located within the N-terminal region and the third within the C-terminal region, located in the Pumilio protein domain. Further validation of the synthesized peptide was performed by mass spectrometric analysis of the peptide sequences and HPLC analysis. Each peptide was injected at 200 μg/animal via the subcutaneous route (2 New Zealand Rabbits each) in Freunds' complete/incomplete adjuvant using a customized 48 day immunization protocol (Genscript). A pre-bleed sample was taken at day−4, with the primary immunization delivered on day 0. Booster immunisations were given on days 14 and 35, test bleeds were taken on days 21 and 42, with the protocol completed on day 48. Polyclonal antibodies were purified from the pooled sera for each of the peptides, and confirmed by ELISA titrations to a dilution limit of 1:512,000 (1.95 ng/ml). Antibodies were purified over a bed of protein A/G coupled beats and concentrated (Using Amicon 30 kDa, Merck) or diluted to the required concentration for use in the different assays they were intended.

In addition, three recombinant proteins were designed and expressed in *E. coli* as GST-tagged fusion proteins, targeting the N-, central polymorphic repeat and C-terminal regions (**Figure 1A**). The N- (nt 67-429) and C-terminal (nt 1513-1698) regions, both showing minimal polymorphism, and the polymorphic central repeat region (nt 430-1512) were PCR amplified from 3D7 genomic DNA. The sequence validated amplified inserts were cloned into the pGEX-2T expression vector (GE Healthcare) followed by additional sequence verification prior to transformation and expression in BL21 (DE3) *E. coli*. Expression and affinity purification were performed as described previously for other GST-fusion proteins (47).

Purified proteins were assessed for purity and integrity by SDS PAGE (**Figure 5A**).

Immunofluorescent Assays and Microscopy

The Protein A/G-affinity purified antibodies raised to the peptide fragments were assessed for reactivity to native parasite proteins by IFA. Synchronized late stage schizonts were smeared, air-dried and fixed with acetone (Merck) for 5 min at RT. The slides were co-incubated with rabbit anti-PfMAAP antibodies (PfMAAP 1-3) (1:500) and either anti-mouse PfAMA1 (1:1,000) (MR4), α-PfRAP2 (1:1,000), α-PfRON4 (1:400), α-PfMSP1 or α-PfGAP45, respectively, for 1 h in 3% BSA/PBS buffer, followed by three washes in 1X PBS at 5 min/wash. The slides were then incubated with Alexa Flour 594 goat anti-mouse IgG (H+L) (red) and Alexa Flour 488 goat anti-rabbit IgG (H+L) (red) secondary antibodies (1:1,000 respectively) (Molecular Probes) for 1 h. Slides were washed in 1X PBS three times for 5 min each and air-dried. Mounting medium containing DAPI (Vector laboratories) was added to each slide and sealed with a coverslip for microscopy. The cells were imaged on an Olympus System Microscope Model BX41 with a Hamamatsu ORCA-spark Digital CMOS camera C11440-36U. In total 50 images were taken for each antibody used. All image background subtraction, brightness and contrast adjustment as well as all analysis were conducted using Fiji ImageJ software.

Western Blot Analysis of Parasite Extracts and Culture Supernatants

Ring stage cultures were synchronized with 5% D-sorbitol (Sigma) and cultured to mature stage schizonts (48, 49), followed by purification over a Percoll gradient (50). The purified schizonts were washed twice with 1xPBS and resuspended in 1xPBS. Aliquots were lysed in SDS PAGE sample buffer, resolved using a 12% SDS-PAGE before transfer onto nitrocellulose membranes (0.2 μm, Bio-Rad). Proteins were detected using the PfMAAP1 & 2 polyclonal antibodies (pAbs) @ 1:1,000 dilution. Following primary incubation, the blots were washed and incubated with anti-rabbit HRP-conjugated secondary antibodies @ 1:3,000 dilution for 1 h followed by two washes with 1X PBS. The membrane was developed using enhanced chemiluminescence (GE healthcare) and developed using the KODAK image analysis system.

Merozoites Invasion Inhibition Assay

Synchronized late-stage schizonts were purified, and assays were plated as previously described (50). Briefly, target cells (erythrocyte acceptor cells) were stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester at 20 μM (5(6)CFDA-SE; Invitrogen), a cytoplasmic fluorescent stain, to help differentiate erythrocytes invaded in the assay from those in the parasite inoculum. Late stage parasites at 2% parasitemia were mixed with the 5(6)CFDA-SE-labeled erythrocyte acceptor cells in a 1:1 ratio at 2% hematocrit in 100 μl assays in 96-well titer plates. Increasing concentrations of purified anti-peptide antibody for PfMAAP peptides 1- 3 (0–500 μg/ml) were added to corresponding wells. Control wells were incubated with pre-immune sera. Assays plates were incubated overnight at 37°C in a mixed gas environment to allow one cycle of invasion. Cells were stained with Hoechst 33342 (Sigma Aldrich) and washed 3 times with complete RPMI. Invaded target cells were counted with a BD LSR Fortessa X20 flow cytometer. The experiment was run twice independently with each condition conducted in triplicate. A total of 50,000 RBCs were counted and % invasion into the 5(6)CFDA-SE-labeled target cells were recorded. The percentage of successfully invaded RBCs in the presence of anti-PfMAAP antibodies or the pre-immune controls were compared with the level of invasion in control wells without antibody added.

Expression Levels of PfMAAP

The expression of the PfMAAP were assayed in three laboratory isolates (3D7, Dd2 and W2mef), following 6, 8-h sample collection time points. The expression analysis assay protocol was as described by Baker et al. (51). Briefly, RNA was purified from tightly synchronized parasites as described previously, using the AllPrep DNA/RNA Mini kit protocol by Qiagen (Qiagen, Germany) and treated with DNase to remove all traces of DNA in the sample. The cDNA synthesis was then carried out with a control reaction (without a reverse transcriptase) using the Superscript III first-strand protocol following manufacturer's instructions (ThermoFisher Scientific, USA). The cDNA synthesis reaction conditions were as follows: 25°C for 10 min, 50°C for 50 min and 85°C for 5 min. Following the cDNA synthesis, 1 μl of RNase H was added to each reaction, mixed and incubated at 37°C for 20 min followed by 95°C for 10 min to remove unconverted RNA molecules. The quantitation of the transcript level of PfMAAP gene was evaluated relative to the 60S ribosomal protein L18-2 (PF3D7_1341300) (51); a housekeeping gene used as a control. Each experiment was conducted in triplicate and conducted at two independent times. The expression levels were calculated from the Ct values using the $2^{-\Delta\Delta Ct}$ formula.

Analysis of Naturally Acquired Antibodies

The plasma samples analyzed in the study were previously collected as part of a community cohort study undertaken in Chonyi, Kenya. A village in Kilifi district near the eastern coast of Kenya. The inhabitants were naturally exposed to biannual peaks of transmission in November to December and May to July, with moderate rates of transmission at the time of sampling (22–53 infectious bites/person/year; October 2000) (33). Active and passive case detection was used to determine the occurrence of episodes of clinical malaria in the following 6-month period within the communities. Indirect ELISAs were performed with each of the three antigens using protocols as previously described (47). Briefly, antigens coated at 50 ng/well were in duplicate with sera diluted to 1/500. Due to the fact that the proteins used in the assay were generated as GST-tagged fusion proteins, purified GST was included in the assay and results subtracted to correct for any background reactivity to the tag. Samples were scored as positive if the ELISA optical density (OD) values were higher than the mean plus 3 standard deviations of the values from 20 malaria-naive control sera tested in parallel (the same panel of

negative-control sera was used in all assays). The risk of clinical malaria and association with antibody status was analyzed for subjects who were asymptomatic and <11 years of age at the time of sampling (Chonyi N=119/518), as done previously in analyses of antibodies to other antigens (47).

Data Analysis

Boxplots were generated in R (R studio, version 3.5.2). Generalized linear models (GLM) were used to determine the risk ratio (RR) associated with the presence or absence of detectable serum antibodies (IgG above the cut-off OD value) and the occurrence of subsequent clinical malaria episodes. Age and antibody reactivity to parasite schizont extract were used in multivariate analyses to correct for the confounding effects of exposure on antibody responses. Statistical analyses were performed using Stata/IC (StataCorp LP, USA).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YA, GA, and KT: conceptualization. YA, GA, KT, DC, and FO: designed experiments. YA, PN, EC-C, FA, EQ, GK, LT, and KT: performed experiments. YA, KT, PN, and FA: analyzed the data. KT, GA, DC, and KM: supervised the study. YA, KT, GA, and DC: wrote the manuscript. All authors read and agreed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00505/full#supplementary-material

Figure S1 | Amino acid sequence alignment of full-length sequences from 16 Plasmodium falciparum isolates for PfMAAP (PF3D7_1035900, PfSD01_100040400, PfML01_100039900, PfKE01_100041100, PfIT_100039800, PfGB4_100040700, PfGN01_100041300, PfTG01_100041000, PfDd2_100041100, PfSN01_100041200, PfKH02_100041200, Pf7G8_100040200, PfHB3_100040200, PfGA01_100041100, PfKH01_100040300, PfCD01_100041000). Alignments were generated using Clustal Omega (Sievers et al., 2011). All sequences were obtained from Plasmodb (https://plasmodb.org/plasmo/).

Figure S2 | Amino acid alignment of full-length sequences from non-malaria isolates for PfMAAP (PF3D7_1035900) aligned with the 3D7 isolate as a reference. Alignments were generated using Clustal Omega (Sievers et al., 2011). *Plasmodium falciparum*, PF3D7_1035900; *Plasmodium rechenowi*, PRG01_1034400 and PRCDC_1035200; *Plasmodium billcolllinsi*, PBILCG01_1034800; *Plasmodium gaboni*, PGSY75_0012400; *Plasmodium praefalciparum*, PPRFG01_1036900; and *Plasmodium adleri*, PADL01_1034600. All sequences were obtained from Plasmodb (https://plasmodb.org/plasmo/).

Figure S3 | N- and C-terminal semi-conserved and armadillo repeat sequences for PfMAAP in **(A)** sixteen laboratory adapted isolates (PF3D7_1035900, PfSD01_100040400, PfML01_100039900, PfKE01_100041100, PfIT_100039800, PfGB4_100040700, PfGN01_100041300, PfTG01_100041000, PfDd2_100041100, PfSN01_100041200, PfKH02_100041200, Pf7G8_100040200, PfHB3_100040200, PfGA01_100041100, PfKH01_100040300, PfCD01_100041000) and **(B)** in five *Laverania* subgenus of *Plasmodium* (*P.rechenowi*, PRG01_1034400 and PRCDC_1035200; *P.billcolllinsi*, PBILCG01_1034800; *P. gaboni*, PGSY75_0012400; *P.praefalciparum*, PPRFG01_1036900; and *P. adleri*, PADL01_1034600. All sequences were obtained from Plasmodb (https://plasmodb.org/plasmo/).

Figure S4 | Co-localization of α-*Pf*MAAP1 and 2 with α-AMA1. **(A)** *Pf*MAAP1 (green) localization pattern relative to *Pf*AMA1 (red) with DAPI (blue) staining for nuclei and the merging of all channels (Merge). **(B)** *Pf*MAAP2 (green) co-localization with *Pf*AMA1 (red) with DAPI (blue) staining the nuclei and the merge of all channels (Merge). Fifty images were taken per antibody tested using an Olympus model BX41 fluorescent microscope with a x100 oil-immersion objective.

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