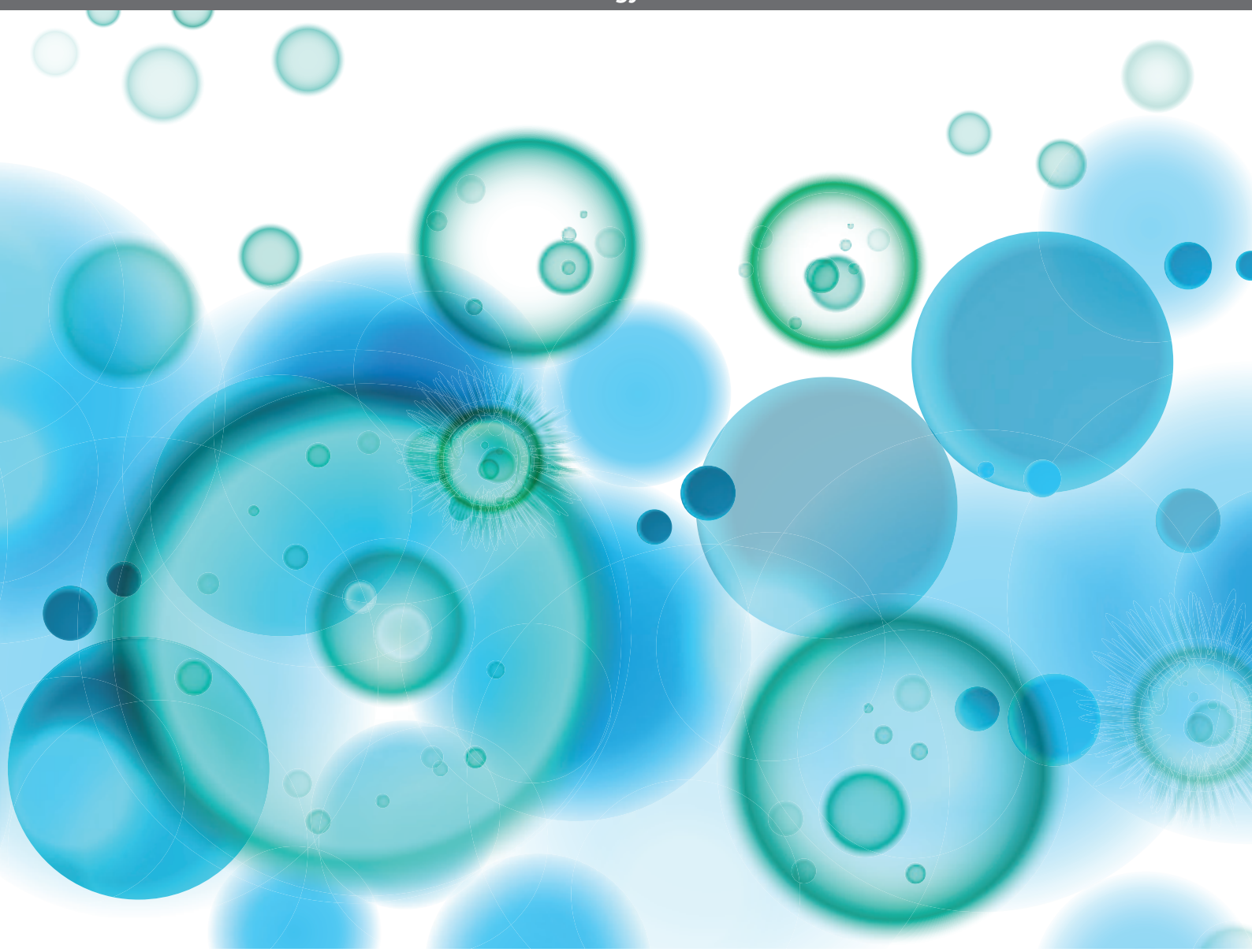
RISING STARS IN PARASITE IMMUNOLOGY 2021

EDITED BY: Pedro H. Gazzinelli-Guimaraes, Anton Götz and Christine Hopp
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-83250-877-0
DOI 10.3389/978-2-83250-877-0

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RISING STARS IN PARASITE IMMUNOLOGY 2021

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Citation: Gazzinelli-Guimaraes, P. H., Götz, A., Hopp, C., eds. (2022). Rising Stars in Parasite Immunology 2021. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-877-0

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frontiers in Immunology

ORIGINAL RESEARCH
published: 04 January 2022
doi: 10.3389/fimmu.2021.787091

The GT1-TPS Structural Domain Protein From *Haemonchus contortus* Could Be Suppressive Antigen of Goat PBMCs

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: 30 September 2021
Accepted: 08 December 2021
Published: 04 January 2022

Citation:
Wen Z, Aleem MT, Aimulajiang K, Chen C, Liang M, Song X, Xu L, Li X and Yan R (2022) The GT1-TPS Structural Domain Protein From Haemonchus contortus Could Be Suppressive Antigen of Goat PBMCs. Front. Immunol. 12:787091. doi: 10.3389/fimmu.2021.787091

Trehalose phosphate synthase (TPS), a key enzyme in trehalose synthesis, is not present in mammals but critical to the viability of a wide range of lower organisms. However, almost nothing is known about the function of Hc-TPS (GT1-TPS structural domain protein from *Haemonchus contortus*). In this study, Hc-TPS gene was cloned and the recombinant protein (rHc-TPS) was expressed and purified. The quantitative real-time PCR (qPCR) results showed that Hc-TPS was transcribed at different stages of *H. contortus*, with higher levels of transcription at the molting and embryo stages. Immunofluorescence analysis showed that Hc-TPS was widely distributed in adults, but the expression was mainly localized on the mucosal surface of the intestine as well as in the embryos of female worms. The impacts of rHc-TPS on peripheral blood mononuclear cell (PBMC) proliferation, nitric oxide (NO) generation, transcriptional expression of cytokines, and related pathways were examined by co-incubating rHc-TPS with goat PBMCs. The results showed that rHc-TPS significantly inhibited PBMC proliferation and NO secretion in a dose-dependent manner. We also found that rHc-TPS activated the interleukin (IL)-10/signal transducer and activator of transcription 3/suppressor of cytokine signaling 3 (IL-10/STAT3/SOCS3) axis and significantly promoted SOCS3 expression, while inhibiting interferon-gamma (INF-γ), IL-4, IL-9, and IL-2 pathways. Our findings may contribute to understanding the immune evasion mechanism for the parasite during host–parasite interactions and also help to provide ideas for discovering new drug targets.

Keywords: ***Haemonchus contortus*, trehalose phosphate synthase, cytokines, PBMCs, immunomodulation**

Abbreviations: GT1-TPS, trehalose phosphate synthase structural domain protein; LB, Luria Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, Ni2+-nitrilotriacetic acid; PVDF, polyvinyl difluoride; TBS-T, Tris-buffered saline containing 0.05% Tween-20; CCK-8, cell counting kit-8; NO, nitric oxide; qPCR, quantitative real-time PCR; IFA, immunofluorescence assay; PBS, phosphate-buffered saline; Th, CD4+ T helper cells; IFN-γ, interferon gamma; IL, interleukin; STAT5, signal transducer and activator of transcription 5; SOCS3, suppressors of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3.

INTRODUCTION

H. contortus is an important pathogenic nematode causing huge economic losses worldwide (1) and is able to coexist with its host under harsh conditions that escape digestion by stomach acid and pepsin. The main control measures for haemonchosis are based on the use of anthelmintics. However, the excessive and uncontrolled use of some antiparasitic drugs has led to widespread parasite resistance over the decades (2). *H. contortus* is characterized by genetic diversity and a multistage life cycle (3, 4), which helps the worm to evade the host's immune system and also contributes to the development of drug-resistant strains (5). Therefore, an in-depth study of key molecules involved in the mechanism of *H. contortus* interaction with the host is expected to identify new drug targets for controlling *H. contortus* infection in the future.

H. contortus is one of the most important parasitic nematodes of considerable economic importance in small ruminants such as sheep and goats (6). In the host–parasite relationship, the parasite excretes and secretes a large number of molecules into the host to regulate the host's immune function. It was shown that *H. contortus* excretory/secretory products (HcESPs) coincubated with PBMCs *in vitro* inhibited the production of IL-4 and IFN-γ, suppressed cell proliferation and nitric oxide secretion, and promoted IL-10 secretion for anti-inflammatory effects (7). A variety of molecules derived from *H. contortus* nematodes have been identified to modulate the function of PBMCs, such as rHc-STP-1 (8) and rHc-TpMy (9), which have unique immunosuppressive effects on goat PBMCs and may be one of the mechanisms that promote immune evasion.

TPS catalyzes the first step in trehalose synthesis (10), which involves the transfer of glucose from uridine diphosphate glucose (UDPG) to glucose 6-phosphate (G6P) to form trehalose-6-phosphate (11). Recent studies have shown that TPS is also involved in the regulation of insect physiology and behavior, including survival, molting, pupal metamorphosis, and chitin metabolism (12–15). In cotton bollworms, it was shown that TPS expression levels were higher in the nymphal stagnation period than in the non-stagnation period and could resist cold during the nymphal stagnation of cotton bollworms (16, 17). Trehalose, catalyzed by TPS, is an important defense mechanism for many pathogens, especially those living in extreme environments (18). Studies have shown that trehalose has specific bioprotective properties that protect organisms in harsh environments (19–24), including drying, dehydration, high acidity, heating, freezing, and oxidation. In nematodes, the concentration of trehalose is usually higher than that of free glucose, which provides energy as the main circulating sugar and is important for egg incubation (25). However, little is known about the role TPS plays in the growth, development, metabolism, and parasitism of *H. contortus*.

This study identifies the transcription of Hc-TPS protein in different developmental phases of the *H. contortus* and localization of expression in the adult stage. In addition, the effect of rHc-TPS on the function of goat PBMCs was assessed.

MATERIALS AND METHODS

Ethics Declaration

Animal experiments were conducted following the guidelines of the Animal Ethics Committee, Nanjing Agricultural University, China. All experimental rules were approved by the Science and Technology Agency of Jiangsu Province. The approval ID is SYXK (SU) 2017-0027.

Animals, Parasites, and PBMC Isolation

Three-to-six-month-old native crossbred goats from the Nanjing Agricultural University research and teaching herd were housed indoors and provided with microbial-free feed with free drinking water. The parasites (*H. contortus*) were maintained through consecutive passages of worm-free goat. Eggs, third-stage larvae (L3s), exsheathed L3s (xL3s), adult males, and adult females were collected as mentioned previously (7, 26). Healthy goats were kept individually in ventilated cages to prevent accidental infection from nematodes and fed them with hay, whole–shell corn, and free freshwater in their enclosures. The blood samples were taken from healthy goats. Afterward, PBMCs were assembled by the gradient centrifugation technique (27).

Female Wistar rats (body weight 250 g) were procured by the Animal Experimental Station of Jiangsu and raised in the Nanjing Agricultural University Experimental Animal Center.

Cloning of Hc-TPS and Expression of rHc-TPS

H. contortus complementary DNA (cDNA) was synthesized as previously described (28). The 160–702-aa region of the glycosyltransferase domain-containing protein (GenBank: HF965754.1) is the 6-phosphorylase synthase structural domain (Hc-TPS). The nucleotides 160 to 702 aa of the glycosyltransferase domain-containing protein were cloned using specific primers (**Supplementary Table 1**). The PCR amplification was carried out using 2 × Phanta Master Mix (Vazyme Biotech, Nanjing, Jiangsu, China). The cyclic terms were used accordingly: the first denaturation for 3 min at 94°C (1 cycle), denaturing (10 s at 94°C), annealing (30 s at 65°C), extension (2 min at 72°C) (30 cycles), and final extension (72°C for 5 min (1 cycle). The PCR product was cleaned by using Gel Extraction Kit (Vazyme Biotech, Nanjing, Jiangsu, China). The Hc-TPS gene was cloned into the pET28a (+) vector (Novagen, Madison, WI, USA) expression plasmid with *Sac* I/*Xho* I restriction sites. Then, the Hc-TPS gene was again sequenced to confirm the right reading frame. The recombinant plasmid (pET28a (+) ligated with Hc-TPS) was again cultured into LB (Luria-Bertani) media used with kanamycin sulfate (optical density at OD600), and the expression was induced into *E. coli* (BL21, DE3) *via* isopropyl-β-D-thiogalactopyranoside (IPTG; working concentration: 1 mM) for 5 h at 37°C (29). The rHc-TPS protein was attached with a histidine-tag and obtained from bacterial lysis *via* His-Trap HP columns (GE Healthcare, Piscataway, NJ, USA), and dialysis was performed by renaturation buffer (20 mmol/l Tris–HCl, 500 mmol/l NaCl, 1 mmol/l GSH, 0.1 mmol/l GSSG, pH 8.0) containing different urea concentrations (8, 6, 4, 2, and 0 M) and using PBS (pH 8.0). The rHc-TPS protein was determined

(size and purity) on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The lipopolysaccharide (LPS) was decontaminated with the rHc-TPS protein by the use of the Endotoxin Removal Kit (GenScript, Nanjing, China), and the concentration was measured by using the BCA kit (Thermo Fisher Scientific, Rockford, USA).

Preparation of Polyclonal Antibodies

The polyclonal antibodies were generated against rHc-TPS. Approximately 300 μg of rHc-TPS protein was mixed 1:1 with Freund's complete adjuvant (Sigma-Aldrich, Shanghai, China) and injected subcutaneously on multiple sites in the Wistar rats. Two weeks later, the rats received three booster doses of the same concentrations of protein with Freund's incomplete adjuvant (1:1, Sigma-Aldrich, Shanghai, China), each 7 days apart. Seven days after the latter booster immunization, rats were anesthetized and anti-rHcTPS antibodies were collected in the serum.

Detection of rHc-TPS by Western Blot Assay

The rHc-TPS protein (30 μg) was separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) as described previously (30). Afterward, the non-specific binding site by skim milk (5%) in Tris-buffered saline solution consisting of 0.1% Tween-20 solution (TBST) was blocked. Subsequently, the membranes were cleaned three times by TBST and incubated with a primary antibody (serum from goat infected with *H. contortus*, 1:100 dilution in TBST) at 4°C overnight. The membranes were again cleaned and incubated by HRP-conjugated rabbit anti-goat IgG (diluted 1:5,000, USA) for 1 h at 37°C. Finally, the blots were revealed by enhanced chemiluminescence (ECL) in an ImageQuant 300 cabinet (GE Healthcare Biosciences, USA) according to the manufacturer's instructions.

Evaluating Transcript Abundance Using qPCR

Transcription of Hc-TPS was observed in different developmental stages (eggs, L3s, xL3s, and adults) of *H. contortus* by qPCR using the primers (HcTPS-F and Hc-TPS-R) (**Supplementary Table 2**). Total RNA was separated from eggs, L3s, xL3s, female adults, and male adults used by the TRIzol method (Vazyme Biotech, Nanjing, Jiangsu, China) according to the manufacturer's protocol. The cDNA was synthesized by the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Nanjing, Jiangsu, China). The β-tubulin gene was utilized as the referring gene. The data were investigated according to raw cycle thresholds (Ct) which were attained from ABI Prism 7500 software (Applied Biosystems, USA) used by the relative Ct ($2^{-\Delta\Delta Ct}$) method. Three separate experiments were carried out with the replicates of each group.

Localization of Hc-TPS in Mature Worms (*H. contortus*)

The adult worms were collected from goat abomasum and placed immediately for fixation into a solution of 4% paraformaldehyde solution for 12 h. Samples were embedded into paraffin wax, and worms (*H. contortus*) were sliced into 4-μm-thick sections using a rotary slicer (Leica, Germany). The slides were processed with citrate buffer (0.01 mol/l) to repair the antigen, and then non-specific binding was blocked with 5% skim milk for 1 h at room temperature. Sections were incubated with primary antibody (rat-anti-rHcTPS antiserum and normal rat serum) at 4°C overnight. Subsequently, three times washed with PBS and incubated with a Cy3-coupled secondary antibody (goat-anti-rat IgG) for 1 h at 37°C. The 4′,6-diamidino-2-phenylindole (DAPI, Beyotime, Nanjing, China) was used to stain the corresponding nuclei within the worm sections, incubated for 7 min, and washed again three times with PBST. In the end, the longitudinal cross section was observed using a laser confocal microscope (LSM 710, Zeiss, Germany).

Binding of rHc-TPS to Goat PBMCs

Freshly isolated goat PBMCs were incubated with rHc-TPS protein (10 μg/ml) and the same volumes of control buffer (PBS) for 1 h at 37°C, respectively. Briefly, cells were fixed in 4% paraformaldehyde on polylysine-coated glass slides and blocked *via* skim milk (5%) for 30 min at room condition. Afterward, the slides were incubated with rat anti-rHc-TPS serum (1:100) at 4°C overnight, cleaned three times with PBS, and again incubated with Cy3-tagged goat anti-rat IgG (1:500) at 37°C for 1 h. Prior to staining with DAPI (Beyotime, Nanjing, China), the nuclei were again washed, and cells were imaged using a confocal laser scanning microscope (LSM 710, Zeiss, Germany).

Cell Proliferation Assay

PBMCs (1×10^6 cells/ml) were incubated with serial concentrations of rHc-TPS (10, 20, 40, and 80 μg/ml) and the same volume of control buffer (PBS) with 5% CO_2 for 24 h at 37°C. The cell proliferation assays were detected by adding 10 μl of CCK-8 solution (Beyotime, Nanjing, China) with another 2-h incubation, after which the OD_{450} (optical density) values were calculated by using a microplate reader (Thermo Scientific, USA). The OD_{450} value of the control was set at 100%, and the following equation was used to calculate the cell proliferation index: OD_{450} sample/OD_{450} control. Three independent trials were conducted with three technical repeats of each group.

Nitric Oxide Production Assay

PBMCs (100 μl, 1×10^6 cells/ml) were inoculated in a DMEM culture medium in 96-well plates. The cells were incubated with rHc-TPS (10, 20, 40, and 80 μg/ml) and PBS as a control (at 37°C and 5% CO_2 for 24 h). The subcellular nitric oxide generation of PBMCs was calculated using a Nitric Oxide Assay Kit (Beyotime, Shanghai, China). Three independent trials were conducted with repeats per group.

Transcriptional Abundance of Inducible Nitric Oxide Synthase, Cytokines, and Related Pathway Molecules Detected by qPCR

Group settings were consistent with the description of nitric oxide production assay. Total RNA extraction and cDNA preparation were performed as previously reported (31). The primers INF-γ, IL-2, IL-4, IL-9, IL-10, IL-17, transforming growth factor β (TGF-β), IL-2R, inducible nitric oxide synthase (iNOS), and signal transducer and activator of transcription 5 (STAT5) for qPCR are listed in **Supplementary Table 2**. The β-actin gene was utilized as a reference gene. The

data were calculated according to raw cycle thresholds (Ct) which were attained from ABI Prism 7500 software (Applied Biosystems, USA) using the relative Ct ($2^{-\Delta\Delta Ct}$) method. Three independent trials were conducted for each group.

The Expression Abundance of IL-10, p -STAT3, and SOCS3 Protein Detection by Western Blot Assay

Group settings were consistent with the description of nitric oxide production assay. Total protein was extracted after washing the PBMCs with PBS, and 70 μl of RIPA modified lysis buffer was added (Beyotime, Nanjing, China). Protein concentrations were determined through the Bradford method used by Bio-Rad Protein Assay (Bio-Rad, USA) reagent and bovine serum albumin (BSA, Sigma-Aldrich Co.) as a standard. Proteins (30 μg) were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) for Western blot analysis. The non-specific binding was blocked with 5% BSA. The membranes were cleaned three times *via* TBST and then incubated with first antibodies (anti-p (Tyr705)-STAT3 (Novus Biologicals, USA), anti-SOCS3 (Beyotime, Nanjing, China), anti-IL-10 (Affinity Biosciences, china), and anti-β-actin (AB clonal, China)) overnight at 4°C. The membranes were again washed three times and incubated with HRP-conjugated goat anti-rabbit IgG (Beyotime, China) at 37°C for 1 h. Finally, the blots were revealed by enhanced chemiluminescence (ECL) in an ImageQuant 300 cabinet (GE Healthcare Biosciences, USA) according to the manufacturer's instructions. The band intensity was analyzed through ImageJ software.

Detection of Nuclear Translocation of STAT3 by IFA

Group settings were consistent with the description of nitric oxide production assay. IFA used the STAT3 polyclonal antibody (1:50, Affinity Biosciences, China) as the primary antibody and goat anti-rabbit IgG coupled to Cy3 as the secondary antibody. This was performed with reference to the binding of rHc-TPS to goat PBMC assay.

Data Analysis

Statistical analyses were performed by using the GraphPad Premier 6.0 software package (GraphPad Prism). The results were presented in terms of mean ± SEM. The Student's t-test was performed to determine differences between the two groups. The differences between the groups were statistically calculated by one-way analysis of variance (ANOVA). p-value < 0.05 was considered as statistically significant.

RESULTS

Cloning, Expression, and Western Blot Analysis of Hc-TPS

The PCR product of Hc-TPS amplification was attained from *H. contortus* by using cDNA with a precise set of primers, and the right segment size of 1,629 bp (**Figure 1A**) of the 543 protein-encoding amino acids was detected. The Hc-TPS gene was effectively cloned into the pET-28a expression vector, and 1,629 bp was verified by enzymatic digestion *via* online BLAST analysis (**Figure 1B**). The recombinant plasmid (pET-28a/Hc-TPS) was induced *via* IPTG and expressed in *E. coli* BL21 (DE3), and rHc-TPS protein was displayed on SDS-PAGE with Coomassie blue staining (**Figure 1C**). Purified rHc-TPS was obtained using Ni2+ affinity chromatography, and a single band with a predetermined fraction of 64 kDa was identified (**Figure 1D**). Moreover, the results of the Western blot showed that rHc-TPS protein was recognized by the goat serum infected with *H. contortus* but could not be recognized by the sera from normal goat (**Figure 1E**). This suggests that Hc-TPS may have potential as a diagnostic antigen or modulator of host immune function.

Localization of Hc-TPS in Adults and Transcription at Different Developmental Stages of *H. contortus*

The transcription of the Hc-TPS gene at various developmental stages of *H. contortus* was examined by RT-qPCR assay, and the β-tubulin gene was used as an internal reference. As shown in **Figure 2A**, Hc-TPS transcript levels were the lowest in the L3 stage and significantly higher in the adult (female and male), xL3, and egg stages compared with the L3 stage, especially in the egg and xL3 stages. In the adult stage, Hc-TPS transcription was significantly increased in females when compared with males. Furthermore, the longitudinal sections of adult male and female *H. contortus* worms were used in IFA to identify the localization of Hc-TPS protein. As shown in **Figure 2B**, Hc-TPS showed a wide distribution in the worm, especially high expression in the intestine of the worm as well as in the embryo of the female (some IFA data not shown).

Effects of rHc-TPS on Different Functions of Goat PBMCs

The binding of recombinant proteins to specific receptors of PBMCs is an important way for them to exert immunomodulation (32, 33). Previous studies in our laboratory have shown that His-tag could not affect the binding of recombinant proteins to PBMCs (31, 34, 35). The binding of rHc-TPS to goat PBMCs was confirmed by IFA. The results showed that the rHc-TPS protein can attach to the cell surface (**Figure 3A**). Moreover, the impact of the rHc-TPS protein on cell proliferation was detected *via* the Cell Counting Assay Kit 8 (CCK8). The proliferation of PBMCs incubated with various doses (10, 20, 40, and 80 μg/ml) of rHc-TPS protein was considerably restrained when compared with the control group (**Figure 3B**). Furthermore, the effects of rHC-TPS on transcript levels of iNOs and NO secretion in PBMCS were examined by qPCR assay and nitric oxide assay kit, respectively. Compared with the control group, different concentrations of rHc-TPS significantly inhibited the transcription of iNOS in PBMCs and showed a dose-dependent relationship (**Figure 3C**). Consistent with expectations, rHC-TPS inhibited NO secretion from PBMC cells in a dose-dependent manner (**Figure 3D**).

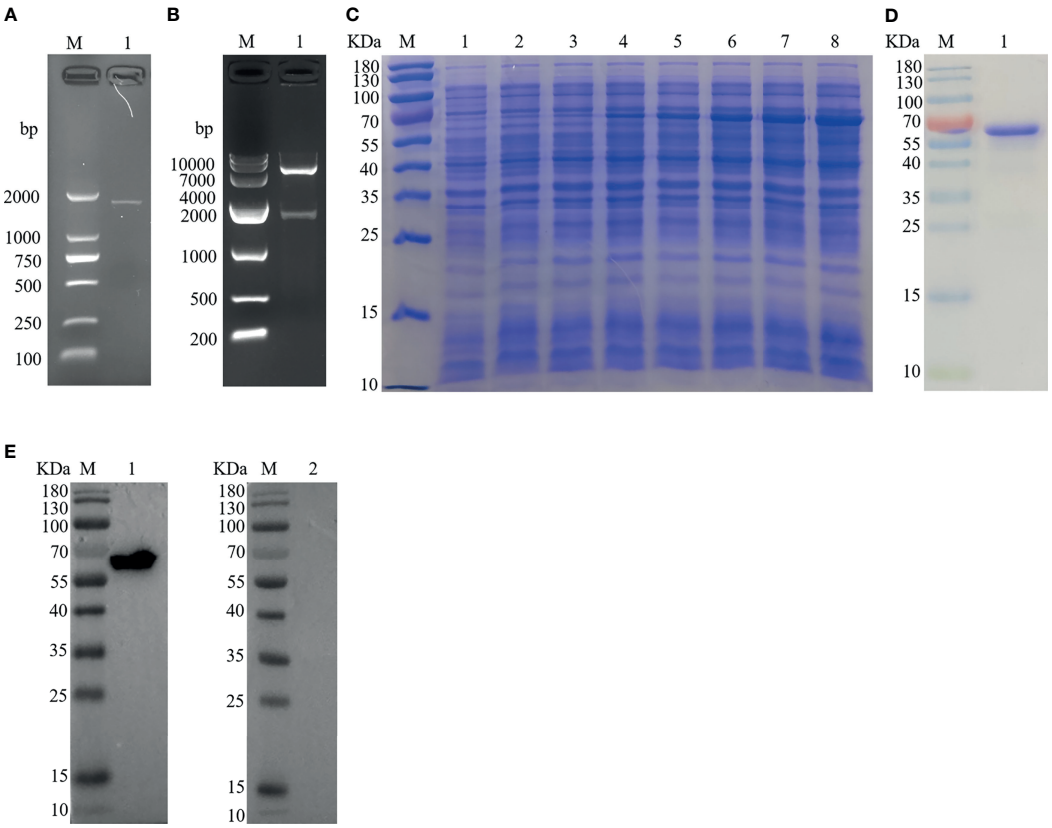

FIGURE 1 | Cloning, expression, and Western blottin analysis of Hc-TPS. **(A)** Amplification of the Hc-TPS gene. Lane M: DNA Marker DL 2000; Lane 1: the amplification products of TPS gene. **(B)** Lane M: DNA Marker DL10000; Lane 1: digestion of pET-28a/Hc-TPS by enzymes. **(C)** The expression of Hc-TPS. Lane M: standard protein molecular weight marker; Lane 1~2: pET-28a induced by IPTG for 0 and 5 h; Lane 3~8: pET-28a/Hc-TPS induced by IPTG for 0~5 (h) **(D)** Lane M: standard protein molecular weight marker; Lane 1: purification of rHc-TPS. **(E)** Western blot examination of rHc-TPS. Lane M: standard protein molecular weight marker; Lane 1: rHc-TPS detected by serum from *H. contortus* experimentally infected goat; Lane 2: reaction with normal goat sera.

Effect of rHc-TPS Protein on the Transcription of Cytokines in PBMCs

Data indicated that there are seven main types of immune responses mediated by T helper cells (Th), including Th1, Th2, Th17, Treg, Tfh, Th9, and Th22 (36). Studies on the immune mechanism of *H. contortus* have focused on Th1 (INF-γ, IL-2), Th2 (IL-4), Th9 (IL-9), Treg (IL-10, TGF-β), and Th17 (IL-17) immune responses. Our aim was to detect the effect of rHc-TPS on cytokines associated with these immune types. The effect of rHc-TPS protein on cytokine production in PBMCs was examined by qPCR assay, which showed that the transcript levels of INF-γ, IL-2, IL-4, and IL-9 were substantially reduced compared with the control group. In contrast, rHc-TPS significantly promoted the transcription level of IL-10 mRNA in PBMCs. However, different concentrations of rHc-TPS had no significant effect on the transcription of IL-17 and TGF-β in PBMCs (**Figure 4**).

Effect of rHc-TPS Protein on the Transcription of the IL-2/STAT5 Signal Pathway

The effect of the rHc-TPS coculture with PBMCs on IL2-R and STAT5 gene transcription was analyzed by qPCR. **Figure 5** displays the effect of rHc-TPS protein on the IL-2/STAT5 pathway, and the result showed that transcript levels of IL-2R and STAT5 were significantly reduced.

The rHc-TPS Protein Activated the IL-10/STAT3/SOCS3 Signaling in PBMCs

Various concentrations of rHc-TPS protein remarkably promoted the transcription of SOCS3 in PBMCs (**Figure 6A**). It also promoted the expression levels of IL-10, p(Tyr705)-STAT3, and SOCS3 in PBMCs (**Figures 6B, C**). IFA results showed that rHc-TPS promotes STAT3 to undergo nuclear translocation (**Figure 6D**).

DISCUSSION

Recent studies have shown that TPS plays an important role in invertebrate growth and development, stress recovery, energy metabolism, chitin synthesis, molting, and other biological processes (11). TPS and trehalose 6-phosphate phosphatase (T6PP) together catalyze the biosynthesis of trehalose, while

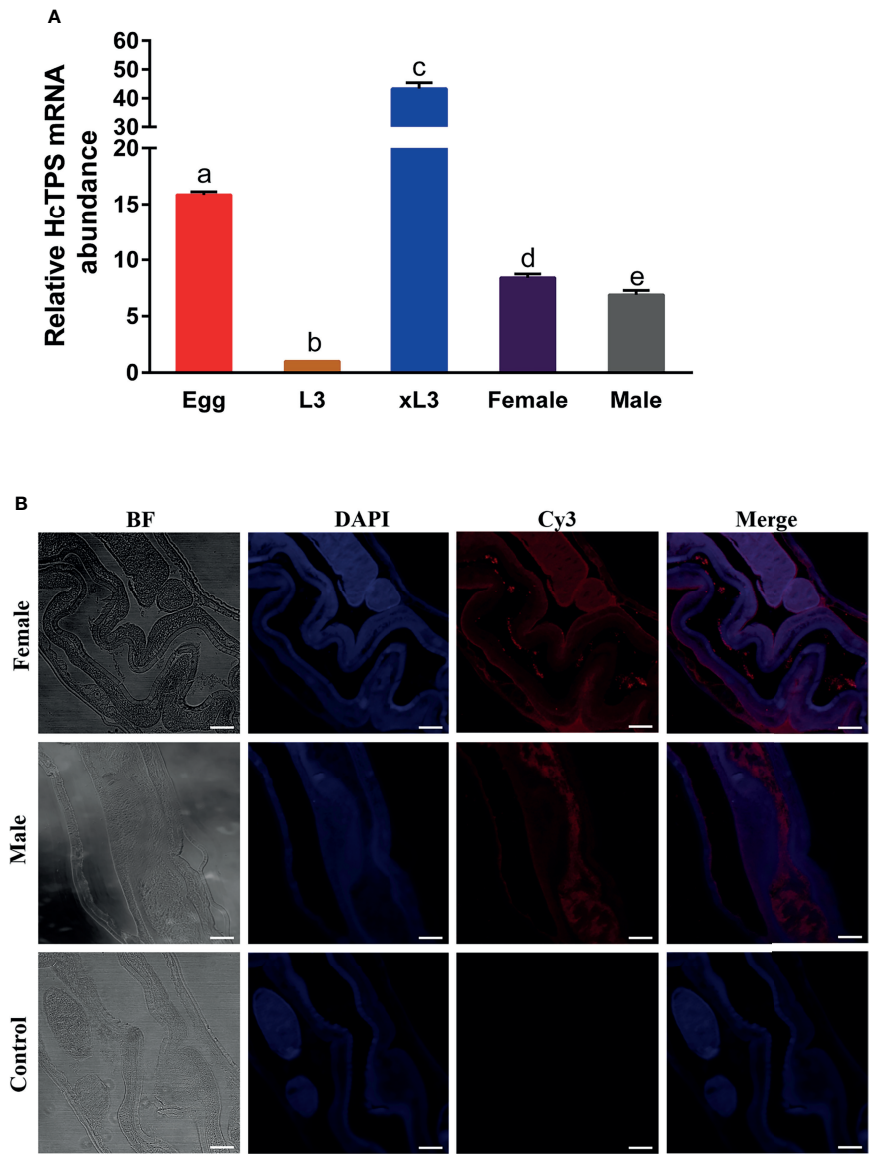

FIGURE 2 | Localization of Hc-TPS in adults and transcription at different stages of *H. contortus*. **(A)** Transcriptional analysis of the Hc-TPS gene in different developmental phases of *H.contortus*. The relative quantities (compared with L3, L3 = 1) are shown as mean values. The results showed here a representative of three independent experiments, and data are represented as mean ± SEM. The Student's t-test was performed to determine differences between the two groups; values without the same letter (a–e) are significantly different ($p < 0.05$). **(B)** Localization of Hc-TPS in adult (male/female) *H. contortus*. Worm sections were incubated with rat-anti-rHcTPS serum and Cy3-coupled goat-anti-rat IgG. DAPI was used to stain the corresponding nuclei. The red color indicates the localization of target protein (Cy3), and the blue color indicates the localization of nuclei (DAPI). No red color was observed in the control (scale-bars: 50 μm).

their synthesis pathways are lacking in mammals. These are promising targets for the development of antibacterial, antifungal, and anthelmintic therapies (37). Our study found that Hc-TPS was transcribed at different developmental phases of *H. contortus*, with relatively high transcript levels in eggs as well as exsheathed L3. In addition, IFA results showed that Hc-TPS was widely distributed in adults, with localized expression mainly on the gut surface and in the embryos of females. TPS transcriptional expression in *H. contortus* was similar to that of trehalose 6-phosphate phosphatase (GOB, unpublished results). The high Hc-TPS gene expression in females as compared to males might be related to the high Hc-TPS expression in the embryo. This implies that we need to further investigate whether Hc-TPS gene silencing or antibody sequestration of the Hc-TPS protein has important effects on larval molting and embryonic development. Western blot results revealed that rHc-TPS protein was identified by sera of goats infected with *H. contortus*, suggesting that Hc-TPS can be identified by the host immune system. In addition, the immunolocalization of Hc-TPS was similar to the *Barbervax*® antigens H-gal-GP and H11 (38, 39),

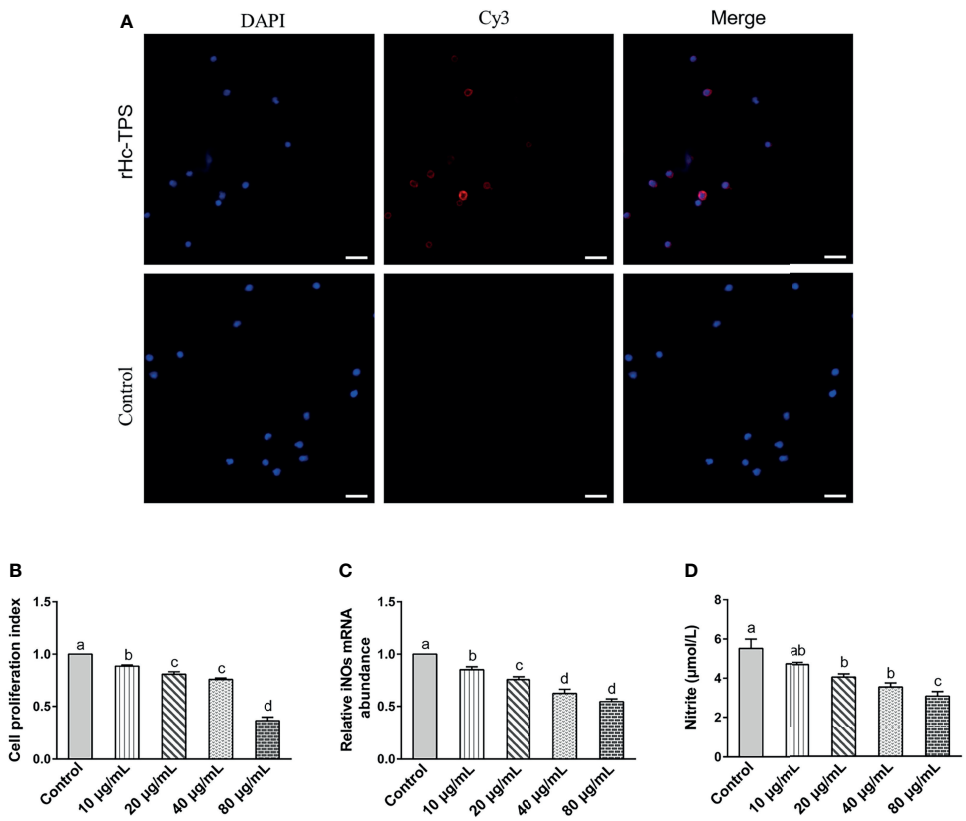

FIGURE 3 | Effects of rHc-TPS on different functions of goat PBMCs. **(A)** Binding of the rHc-TPS protein to PBMCs. rHc-TPS protein was incubated with goat PBMCs, and the binding was detected by rat anti-rHc-TPS serum and Cy3-labelled goat anti-rat IgG. DAPI was used to stain the corresponding nuclei of PBMCs. No red color was observed in the control (scale bars: 20 μm). **(B)** Impacts of rHc-TPS on PBMC proliferation. Cells were incubated *via* various doses of rHc-TPS protein, PBS (control), and the cell proliferation index was calculated by setting the OD_{450} values with the control group as 100%. **(C, D)** Effect of rHc-TPS on nitric oxide production and iNOS transcription by PBMCs *in vitro*. Cells were incubated with serial concentrations of rHc-TPS protein or PBS (control) for 24 h at 37°C and 5% CO_2. Data are presented as the mean ± SEM from three independent experiments, with three technical replicates per group. "a, b, c, or d" based one-way ANOVA analysis to indicate the significance, values without the same letter (a–d) are significantly different ($p < 0.05$).

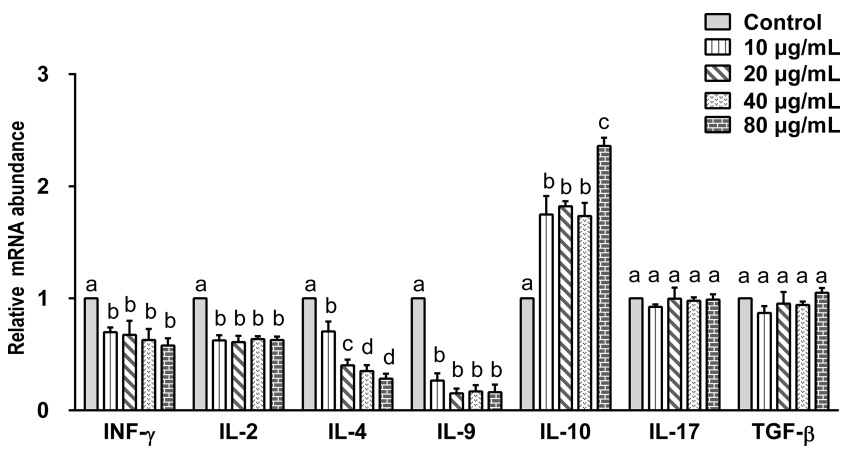

FIGURE 4 | Impact of rHc-TPS protein on the transcription of cytokines in PBMCs. Cells were incubated with serial concentrations of rHc-TPS protein or PBS (control) for 24 h at 37°C and 5% CO_2. The transcription of INF-γ, IL-2, IL-4, IL-9, IL-10, IL-17, and TGF-β were tested by qPCR. The data were presented of three independent trials (mean ± SEM). "a, b, c, or d" based one-way ANOVA analysis to indicate the significance; values without the same letter (a–d) are significantly different ($p < 0.05$).

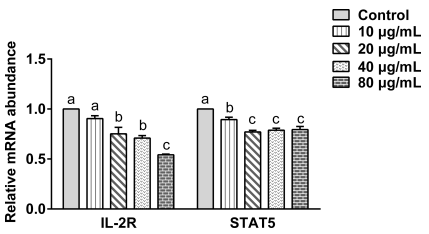

FIGURE 5 | Effect of rHc-TPS protein on the transcription of the IL-2/STAT5 signaling pathway. Cells were incubated with serial concentrations of rHc-TPS protein or PBS (control) for 24 h at 37°C and 5% CO_2. The data were presented of three independent trials (mean ± SEM). "a, b, or c" based one-way ANOVA analysis to indicate the significance; values without the same letter (a–c) are significantly different ($p < 0.05$).

which were locally expressed on the intestinal mucosal surface of *H. contortus*, suggesting that Hc-TPS was more susceptible to attack by host peripheral blood circulating antibodies and may be a vaccine candidate.

When stimulated by antigens, naïve T cells were activated by antigen-presenting cells, and adaptive immune responses were stimulated by cytokine secretion and cell proliferation (40). It has been shown that the ESP of *Brugia malayi* inhibit the proliferation of T lymphocytes, which may contribute to the inability in eliminating the parasite (41–43). In the present study, rHc-TPS protein was found to significantly inhibit the proliferation of PBMCs and showed a dose-dependent manner. This indicated that Hc-TPS could be a suppressive antigen of goat PBMCs. Our results were consistent with previous studies (7, 9). NOS catalyzes the creation of NO, citrulline from L-arginine, oxygen, and cofactors. NO is the product of activation of immune cells by cytokines, microorganisms, parasites, etc. (44). It acts as an antibacterial, antitumor, and parasiticidal agent studied *in vitro* as well as *in vivo* (45, 46). Our study showed that rHc-TPS significantly inhibited the transcription of iNOs and the secretion of NO in PBMCs.

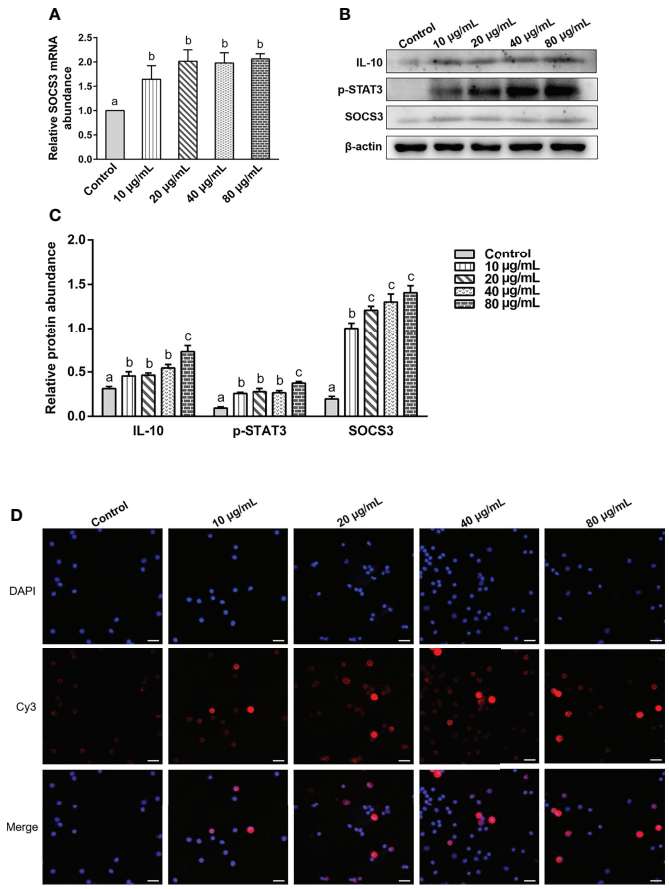

FIGURE 6 | The rHc-TPS protein activated the IL-10/STAT3/SOCS3 signaling in PBMCs. Cells were incubated with serial concentrations of rHc-TPS protein or PBS (control) for 24 h at 37°C and 5% CO_2. **(A)** Transcription study of SOCS3 in goat PBMCs. **(B)** The expression of IL-10, p705-STAT3, and SOCS3 were detected by Western blot. **(C)** Statistics of the IL-10, p705-STAT3, and SOCS3 Western blotting results. The data were presented of three independent trials (mean ± SEM). "a, b, or c" based one-way ANOVA analysis to indicate the significance, values without the same letter (a–c) are significantly different ($p < 0.05$). **(D)** Detection of nuclear translocation of phosphorylated STAT3 by IFA; the red color shows the specific protein localization stained *via* Cy3 on PBMCs, and the blue color indicates the nuclei *via* DAPI. Merged picture shows both DAPI and Cy3. Scale bars: 20 μm.

Previous studies showed that Th1, Th2, Th9, and Th17 immune responses play important roles in fighting parasitic infections (47–50). Our results showed that rHc-TPS co-incubation with PBMCs significantly inhibited the transcriptional levels of IFN-γ, IL-4, and IL-9, which may exert one of the pathways of immune evasion. IL-2 produced by antigen or mitogen-stimulated helper T-cells was one of the most indispensable immunomodulatory factors in immune regulation (51, 52). IL-2 signaling acts through STAT5 and affects the differentiation of helper T-cell subsets (including Th1, Th2, Th9, and Th17 cells), as well as Treg cells (51). IL-2/STAT5 positively regulates the differentiation of Th1, Th2, and Th9 cells; however, it exerts a negative regulatory effect on the differentiation of Th17 cells. In the present study, rHc-TPS was found to significantly inhibit the IL-2/STAT5 pathway, as described in previous studies (53). Thus, rHc-TPS inhibited the transcription of IFN-γ, IL-4, and IL-9 in PBMCs cells, possibly acting through inhibition of the IL-2/STAT5 axis.

IL-10 is a cytokine with anti-inflammatory properties that plays a central role in infection by limiting the immune response to pathogens and preventing the host from overactive inflammatory responses (54). STAT3 is a key effector molecule for IL-10 activation and results in upregulation of SOCS3, which is necessary for IL-10-regulated anti-inflammatory effects (55–59). Our study found that co-incubation of the Hc-TPS protein with PBMCs significantly promoted the transcriptional expression of the anti-inflammatory factor IL-10. Further studies revealed that rHc-TPS significantly activated STAT3, thereby promoting the transcriptional expression of SOCS3. Activation of the IL-10/STAT3/SOCS3 axis by rHc-TPS may be necessary for immune evasion in *H. contortus*.

CONCLUSIONS

In conclusion, the Hc-TPS gene was transcribed at high levels in egg and xL3 stages and was highly expressed in the gut of the adult stage. rHc-TPS could activate the IL-10/STAT3/SOCS3 axis to exert anti-inflammatory effects, and inhibit the proliferation of PBMCs and the secretion of the pro-inflammatory factor NO, while suppressing the transcription of IL-2, IL-4, INF-γ, and IL-9. However, further studies are required to examine the potency of rHc-TPS protein in the goat infection models. Our findings could assist to provide insight into the molecular mechanisms of this protein under host–parasite interactions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Animal experiments were conducted following the guidelines of the Animal Ethics Committee, Nanjing Agricultural University, China. All experimental rules were approved by the Science and Technology Agency of Jiangsu Province. The approval ID is SYXK (SU) 2017-0027.

AUTHOR CONTRIBUTIONS

Data curation, ZW and MA. Formal analysis, ZW and MA. Funding acquisition, RY. Investigation, KA. Methodology, ZW, CC. Project administration, RY. Resources, ZW and MA. Software, ZW and KA. Supervision, XS, LX, XL, and RY. Visualization, ML. Writing—original draft, ZW. Writing—review and editing, ZW, MA, and RY. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the National Natural Science Foundation of China (31872464), the policy guidance project of Jiangsu Province for international scientific and technological cooperation (BZ2019013), the National Key Research and Development Program of China (2017YFD0501200), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

ACKNOWLEDGMENTS

We gratefully thank Shuping Yan from Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture, College of Veterinary Medicine, Nanjing Agricultural University, for her valuable suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.787091/full#supplementary-material

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REVIEW
published: 03 February 2022
doi: 10.3389/fimmu.2022.791006

Gnawing Between Cells and Cells in the Immune System: Friend or Foe? A Review of Trogocytosis

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Trogocytosis occurs when one cell contacts and quickly nibbles another cell and is characterized by contact between living cells and rapid transfer of membrane fragments with functional integrity. Many immune cells are involved in this process, such as T cells, B cells, NK cells, APCs. The transferred membrane molecules including MHC molecules, costimulatory molecules, receptors, antigens, etc. An increasing number of studies have shown that trogocytosis plays an important role in the immune system and the occurrence of relevant diseases. Thus, whether trogocytosis is a friend or foe of the immune system is puzzling, and the precise mechanism underlying it has not yet been fully elucidated. Here, we provide an integrated view of the acquired findings on the connections between trogocytosis and the immune system.

Keywords: trogocytosis, information transmission, immune defense, immune escape, immune regulation

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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *07 October 2021*
Accepted: *14 January 2022*
Published: *03 February 2022*

Citation:
Zhao S, Zhang L, Xiang S, Hu Y, Wu Z and Shen J (2022) Gnawing Between Cells and Cells in the Immune System: Friend or Foe? A Review of Trogocytosis. Front. Immunol. 13:791006. doi: 10.3389/fimmu.2022.791006

TROGOCYTOSIS

Trogocytosis, a form of cell-to-cell interaction widely existing in a species or between different species, involves one cell contacting and quickly "biting" another cell. This interaction was first described in 1970 as part of the process of parasites attacking and killing host cells (1). In 2002,it was given its name from the ancient Greek word "*trogo*", which means "nibbling" to describe the phenomenon of the transfer of membrane fragments containing membrane-anchored proteins between cells (2).

Recently, an increasing number of studies have shown that trogocytosis plays a vital role in the immune system, including antigen presentation, T cell differentiation, immune regulation, and anti-infection and anti-tumor immunity (3–19). Many types of proteins are transferred between cells by trogocytosis, including MHC (major histocompatibility complex) molecules, costimulatory molecules, adhesion molecule receptors, tumor antigens, and the antigens of pathogens (9, 18, 20–27). The involved cell types include T cells (γδ T cells, and $CD4^+$ and $CD8^+$ αβ T cells), B cells, NK cells, dendritic cells, monocytes/macrophages, neutrophils, endothelial cells, fibroblasts, eosinophils, basophils), tumor cells, and pathogen cells (e.g., viruses, bacteria, and parasites) (9, 18, 20–28). Trogocytosis is strictly contact-dependent between living cells. Previous studies have shown that MHC I or HLA-C molecules on the surface of target cells of mice or humans are bidirectionally exchanged with the inhibitory receptor KIR (NK cell Ig-like receptor) of NK cells within a few minutes after coculture by immunological synapses (29, 30). In addition, Ralston et al. showed that amoebae only gnawed live-cell targets and directly

engulfed dead cell corpses, which is one of the characteristics of trogocytosis (31). Interestingly, the membrane molecules obtained by trogocytosis, such as human leukocyte antigen (HLA)-C, peptide-MHC complexes and inhibitory natural killer cell receptors, can be recolonized on the surface of trogocytosis-positive cells without proteolytic cleavage and perform corresponding functions (29, 30, 32).

The outcomes of trogocytosis, which vary with recipient cells, have changed our cognition of some classical theories. Trogocytosis does not always lead to the death of target cells. The interaction is relatively mild and tends to take up material and exchange information under physiological conditions, while it is often strong and usually ends in the death of target cells under pathological conditions (14, 20, 33). The existence of trogocytosis has shaken the traditional notion that cells can only perform their inherent functions or that a gene must be transcribed to use its protein product. With trogocytosis, even mature cells can perform a variety of unconventional functions, and these functions strictly depend on the cellular environment; that is, trogocytosis leads to very different functional consequences with similar processes in different states (34). However, is its function ultimately good or bad? From different perspectives, the role of trogocytosis also shifts between friend and foe in different environments. The reported physiological processes and related diseases involved in trogocytosis are summarized, divide into two aspects (i.e., friend and foe) and shown in **Figure 1**. Specifically, "Friend" refers to the biological events that trogocytosis is beneficial to appropriately enhancing human immunity and improving the ability to resist diseases, whereas "Foe" means the biological events that trogocytosis promotes the occurrence and development of diseases and harms human health.

TROGOCYTOSIS IN THE IMMUNE SYSTEM

The Immune System Transmits Signals Among Cells by Trogocytosis

The communication of immune cells involves various mechanisms, including immune synapses, nanotubes, trogocytosis, and exosomes. The transfer of membrane proteins

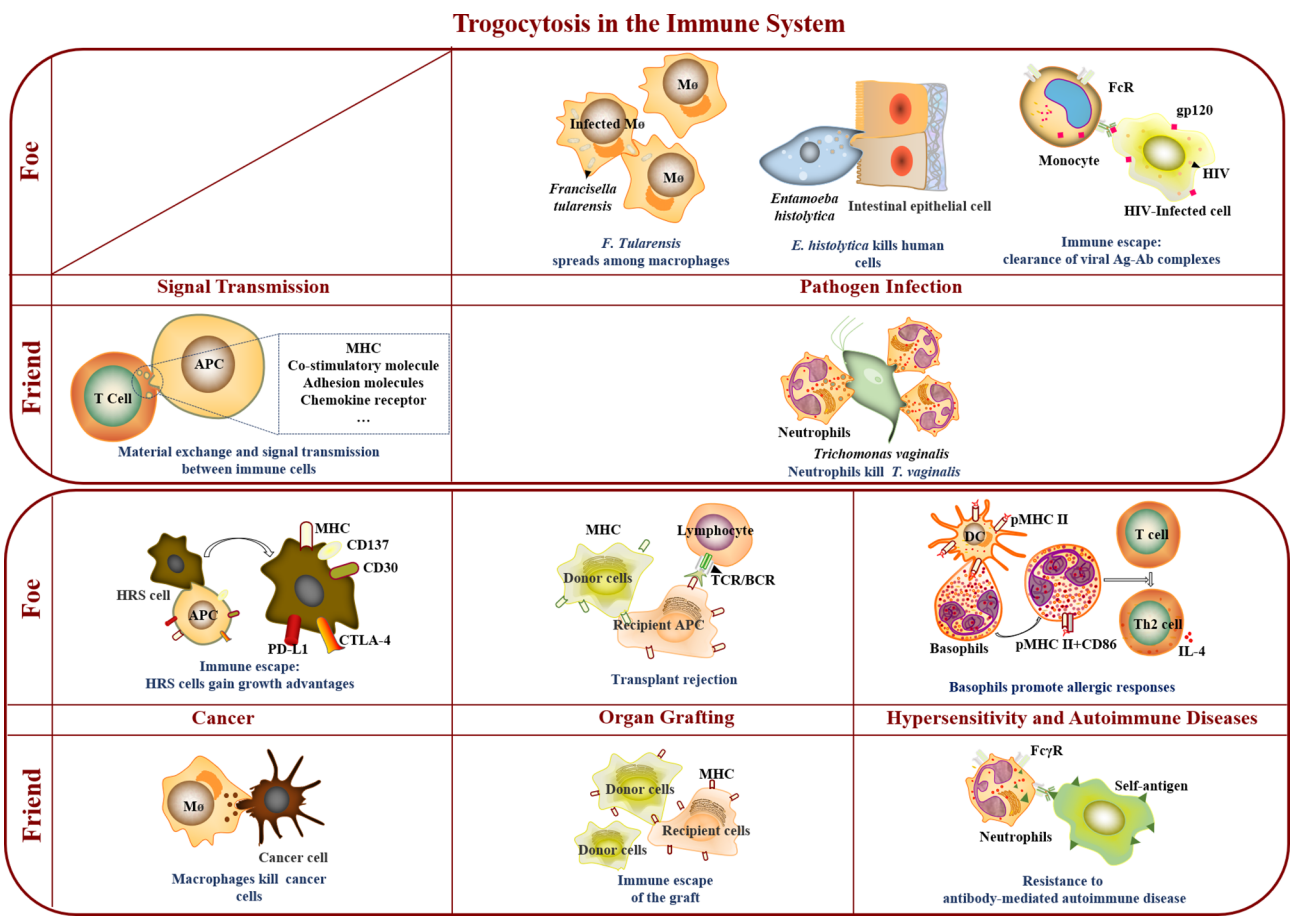

FIGURE 1 | Trogocytosis in the immune system. In the immune system, trogocytosis mediates material exchange and signal transmission between immune cells, participates in immune cell killing of *T. vaginalis* and tumor cells, assists immune escape of grafts, and promotes resistance to antibody-mediated autoimmune disease. As a foe, trogocytosis accelerated *F. Tularensis* spreading among macrophages, and was involved in *E. histolytica* killing human cells, immune escape of virus and tumor cells, transplant rejection, and promotion of allergic responses.

of cells mediated by trogocytosis, including MHC molecules, CD3, costimulatory molecules, endothelial cell molecules, NK receptors, chemokine receptors, and glycosylphosphatidylinositol (GPI)-anchored proteins, widely occurs in T cells, B cells, macrophages, DC, NK cells. The interaction has modified the functions of immune cells involving in antigen presentation, information transfer, and regulation and homeostasis (3, 22, 25, 35–42).

In general, the typical manner for antigen presentation is that professional APCs (such as DCs) process the protein antigens into pMHCs (antigenic peptide-MHC complexes) and then present them to T cells. Some researches revealed a new antigen presentation pathway in which APCs and non-APCs directly obtained preformed pMHCs on the surface of target cells by trogocytosis and activated T cells without further processing (7, 10, 14, 19). Subsequently, the trogocytosis of MHCs occurs not only in T cell-APCs but also in T cell-endothelial cells, APC-APCs, APC-NK cells, tumor cell-T cells, and NK cells (43–47). For example, the transfer of pMHCs and CD80 from APCs to T cells could regulate T cell proliferative signals and sustain their activation in the absence of APCs (32, 48–50). At later time points, the number of activated $CD4^+$ T cells trogocytosing and capturing MHC-peptide complexes increases and outnumbers the APCs, there is increasing probability that newly arriving T cells (and any T cells that have just been activated and need a second hit to continue to divide) would encounter them before encountering a proper professional APC. This T-T interaction leads to the inhibition of the newly arriving T cells and the Ag-experienced T cells, whereas naive T cells encountering antigen-presenting T cells are not inactivated, rather they are activated (51). In addition, Miyake et al. found that basophils gnawed DCs to obtain membrane fragments containing pMHC II and stimulated the proliferation of peptide-specific T cells, which indicated that trogocytosis could enhance the antigen presentation ability of basophils (52).

$CD4^+$ T cells can obtain not only MHCs or pMHCs from APCs by trogocytosis but also costimulatory molecules (CD28, CD54, CD80) from APCs to deliver costimulatory signals to activate $CD4^+$ T cells (48, 53, 54). Zhou et al. showed that MHC II and CD80 in $CD4^+$ T cells maintained the activation of T cells in the absence of APC, which was an important factor in maintaining the homeostasis of memory T cells (50). Interestingly, the trogocytosed molecules might be unmodified in the recipient cell to perform their corresponding function. For example, Reed et al. reported that trogocytosis-mediated signaling has the potential to uniquely modulate the effector-cytokine production and differentiation of $trog^+$ $CD4^+$ T cell after separation from APC. To be more specific, trogocytosis-mediated intracellular signaling in $CD4^+$ T cells drove Th2-associated effector cytokine production and differentiation (15). They found that trogocytosed molecules (pMHC complexes and costimulatory molecules) on $trog^+CD4^+$ T cells engaged their cognate receptors and drove the expression of IL-4 and GATA-3 in sequence, which was consistent with the differentiation of helper T cells type 2 (Th2) (6, 15, 16). Furthermore, extended trogocytosis-mediated signaling in $CD4^+$ T cells resulted in the expression of Bcl-6, programmed cell death protein 1 (PD-1), CXCR5, and IL-21, which was consistent with the differentiation of follicular helper T cells (Tfhs) (5, 16). At the same time, IL-21 promoted the activation and survival of T cells and the generation of memory cells (55–59). On the other hand, trogocytosis might also affect the function of donor cell by removing some molecules from the donor cells. Qureshi et al. show that CTLA-4-expressing cells captured and removed CD80/CD86 from opposing cells to result in impaired costimulation *via* CD28, which revealed a mechanism of immune regulation whereby CTLA-4 acts as an effector molecule to inhibit CD28 costimulation by the cell-extrinsic depletion of ligands (60).

In addition to the transmission of costimulatory signals, trogocytosis also mediates other signals to be transmitted between different immune cells, which maintains immune homeostasis by balancing the activation and suppression of immune responses. It was recently reported that antigen-specific Treg cells form strong interactions with DCs to acquire DC-derived membranes by a process of trogocytosis, resulting in selective depletion of the complex of cognate pMHC II from the DC surface, reducing the capacity of DCs to present antigens (3). The strong binding of Tregs and their capacity to debilitate DC function in an antigen-specific manner, represents a novel pathway involving trogocytosis for Treg–mediated suppression and may be a mechanism by which Treg cells maintain immune homeostasis (3, 61). Trogocytosis also participates in the immunosuppressive effect mediated by Tregs in other ways. Tekguc et al. showed that Treg-expressed CTLA-4 depleted CD80/CD86 by trogocytosis and released free PD-L1 on APCs, which led to dual suppressive effects on T cell immune responses by limiting CD80/CD86 costimulatory naïve T cells and by increasing free PD-L1 available for the inhibition of effector T cells expressing PD-1 (17). Moreover, T cell microvilli-derived particles (TMPs) carrying T cell receptors (TCR) at all stages of T cell activation were separated from T cells by trogocytosis or membrane budding, which were deposited at the surface of cognate APCs to be a potentially effective pathway to transmit information on T cells to APCs (9).

It is reported that human $CD8^+$ T cells played a regulatory role in the immune response by obtaining inhibitory molecules from APCs (62). Studies have shown that human $CD8^+$ T cells obtained functional programmed death-ligand 1 (PD-L1) from APCs in an antigen-specific manner, which led to the apoptosis of neighboring T cells with the expression of the receptor of PD-1 (62). Gary et al. reported that the inhibitory molecule PD-L1 and the receptor PD-1 expressed on various human cells were transferred between immune cells by trogocytosis to regulate the immune response and recycle the molecule (62).

In addition to T cells, the antigen presentation and information transfer by trogocytosis also occurs in B cells. Soluble antigens can be bound to the B cell antigen receptor (BCR), and then internalized and presented to T cells by B cells, which initiates the humoral immune response. However, antigens are often insoluble or tethered to the cell surface. It has been reported that B cells can extract and present antigens that is tethered tightly

to a noninternalizable surface and the evidence points to the major role being played by BCR-mediated wrenching of the antigen from its tether (63). Notably, a weak BCR can apparently wrench a tightly tethered antigen from the plate through continuous accumulation of effect in cases where the affinity difference is of several orders of magnitude (63). The nonstatic BCR antigen tether interaction and the motile nature of the cell may cause distortion of the antigen (and diminution of antigen tether affinity) (63). In addition, Xu et al. found that the BCR interacted with antigen-antibody complexes to remove epitopes from red blood cells by trogocytosis so that IgG could mediate the inhibitory effect on the immune response to antigens (27).

Furthermore, the trogocytosis also affects the functions of macrophages, DC, and NK cells. The trogocytosis by T cells is usually a process of the acquisition of antigens and signal transmission. In addition to the above functions, the trogocytosis by monocytes also involves a process of removing antigens, which show that the acquirer cell may control the functional outcome of trogocytosis (38). It was reported that the trogocytosis by macrophages mediated by FcγR affects the function of target cells, such as T cells and NK cells, but does not obtain new proteins or new functions (41). It is reported that KIR^+ NK cells generally did not express CCR7; however, they were able to extract CCR7 from $CCR7^+$ cells by trogocytosis to migrate to the site of killing mature DCs and T lymphoblasts with the help of chemokines CCL19/CCL21 (39, 40, 42).

Thus, the results showed that trogocytosis was widely involved in antigen presentation, information transfer, and regulation (activation, suppression, and even killing) of immune cells in the immune process, which was of greatly significance to the performance and homeostasis of immune function. Regrettably, the research on whether trogocytosed molecules undergoes degradation, modification and other processes before performing subsequent functions is so lacking that we know very little about this important issue. In addition, how cell membrane integrity is restored post trogocytosis is still a meaningful and not fully recognized process. In summary, our understanding of trogocytosis remains at a relatively superficial level and there are still many unknown fields waiting for us to explore, which also points out the direction for future research.

Immune defense in Tumorigenesis and Pathogenic Microorganism Infection by Trogocytosis

According to the available literature, the trogocytosis by many immune cells, such as neutrophils, macrophages, and NK cells, played an essential role in immune defense in tumorigenesis and pathogen infection by destroying the membrane integrity of pathogens and tumor cells, which led to the loss or disability of organelles and the death of target cells (4, 11–13, 64).

Neutrophils Kill *Trichomonas vaginalis* by Trogocytosis

Previous studies have reported that the mechanisms of neutrophil clearance of *Trichomonas vaginalis* (*T. vaginalis*) included phagocytosis, toxic particles, and NETosis. Interestingly, Mercer et al. recently revealed that human neutrophils could successfully trogocytose *T. vaginalis* to achieve pathogen killing (12, 13). 3D and 4D live imaging showed that neutrophils rapidly surrounded and trogocytosed *T. vaginalis* before parasite death after coculture *in vivo*. *T. vaginalis* could only survive for approximately 8 minutes after trogocytosis started. During this process, the parasite experienced approximately 3 to 8 "bites" by an average of 3 to 6 neutrophils before parasite death. They also found that the trogocytosis by neutrophils and parasite killing depended on the presence of neutrophil serine protease and human serum factors, which reflected the synergistic effect of trogocytosis and toxic particles on parasite killing (12, 13). Furthermore, Leka et al. reported that complement receptor (CR) 3, which is known to bind iC3b, leading to phagocytosis, plays a role in mediating this trogocytosis of *T. vaginalis* (4). *T. vaginalis* is a unicellular parasite. When the membrane fragments removed from the parasite by trogocytosis are sufficient, or the membrane loss exceeds the repair capacity of *T. vaginalis*, the parasite will rupture and die. At this time, trogocytosis helps host to eliminate pathogens, which can be regarded as "Friend". However, whether the trogocytosis by neutrophils or other immune cells can kill other multicellular pathogens, much larger than immune cells, merits future discussion.

Immune Cells Kill Tumor Cells by Trogocytosis

Recently, Matlung et al. reported that antibody-mediated neutrophil trogocytosis killed tumor cells in a contact-dependent manner, which did not release toxic particles and produced reactive oxygen species but only nibbled the plasma membrane of cancer cells, leading to a lytic type of cancer cell death. This mode of destruction of antibody-opsonized cancer cells by neutrophils, called trogoptosis, could be improved by inhibiting the CD47-SIRPα checkpoint (11). Similarly, Steele et al. showed that the continuous trogocytosis by macrophages could kill HER2-overexpressing breast cancer cells, which revealed that the trogocytosis mediated by macrophages led to the death of antibody-opsonized tumor cells (18). In addition, it was reported that human NK cells and T cells obtained the inner membrane protein $H\text{-}RAS^{G12V}$ from tumor cells by trogocytosis, which induced the phosphorylation of extracellular regulated protein kinase ERK and promoted the secretion of INF-γ and TNF-α, the proliferation lymphocyte, and the efficiency of NK cells killing tumor cells (64). Moreover, Joshua et al. reported that when T cells acquire the surface molecules HLA-G and CD86 by trogocytosis, they differentiate into newly acquired Tregs to inhibit the escape of myeloma cells (8). The above example revealed that trogcytosis as a friend of host to suppress tumors.

In short, the emergence of trogocytosis by immune cells has deepened our understanding of antigen presentation, information transmission, and immune regulation and broadened the understanding of immune defense in the immune system. Trogocytosis, evidence of plasticity of the immune system, seems to have impacted traditional immunological theories, which suggests that immune cells can obtain functional molecules beyond their protein

expression profile to perform functions independent of their main characteristics. For example, $CD8^+$ T cells can obtain pMHC II, while $CD4^+$ T cells can obtain pMHC I (48, 65). To some extent, the molecular expression profile on the surface of trogocytosis-positive cells has undergone subtle changes to temporarily display unconventional molecules. However, LeMaoult et al. suggested that APC-like T cells that arose *via* trogocytosis might not functionally compete with the professional APCs from which they took pMHCs and thus might not contribute to the immune response. Similarly, regulation of T-cell proliferative signals by acquired B7 molecules might be of more significant because it provides added value to an immune response by directly affecting the biology of the cell that has acquired CD80 (34). It was reported earlier that although trogocytosis might be an immune response booster from a quantitative standpoint, it should not induce qualitative changes (i.e., changes in the repertoire or function of the antigen-selected cells) and should not have a dramatic impact on immune responses (34). However, according to the previous studies, we can find that trogocytosis has extensively and deeply affected many aspects of the immune system, improving or reshaping people's cognition of antigen presentation, information transmission, immune regulation, and immune defense.

TROGOCYTOSIS IN DISEASES

Immune Escape

Trogocytosis plays a bidirectional role in the immune system. On the one hand, the immune system uses it to defend against tumors and kill pathogens. On the other hand, abnormal cells and pathogens also escape immune system surveillance with the help of trogocytosis. Therefore, trogocytosis plays the dual role of "foe" or "friend" to maintain the homeostasis of the host and pathogens through a long-term balance in immune defense and immune escape.

It is reported that trogocytosis was significantly associated with spread of intracellular pathogens in mice, suggesting that direct bacterial transfer frequently occurs by this process *in vivo (*26). Intracellular pathogens need to enter host cells to replicate. Pathogens must continue invading new target cells to survive and reproduce. During this process, pathogens may be exposed to the extracellular environment containing antibodies, complements, and other inhibitory factors, limiting pathogen spread. However, many reports have revealed that trogocytosis accelerates the spread of intracellular bacteria. Steele et al. found live *Francisella tularensis* (*F. tularensis*) and *Salmonella enterica* (*S. enterica*) were transferred from infected macrophages to uninfected macrophages using trogocytosis with the donor and recipient cells remaining intact, and then *F. tularensis* acquired from infected cells were found within double-membrane vesicles partially composed from the donor cell plasma membrane (26, 66).

Trogocytosis also plays an important role in promoting virus infection. During H5N1 influenza virus infection, B cells obtained the avian flu receptor of α2,3 sialic acids from monocytes using trogocytosis to increase their susceptibility to H5N1 virus infection (67). From another perspective, H5N1 influenza virus might utilize trogocytosis to expand its cell tropism and spread to immune cells with the lack of avian flu receptor (67). During HIV infection, monocytes can remove well-exposed antibody-gp120 complexes from the surface of infected cells through antibody-mediated trogocytosis, thereby evading surveillance of the immune system (21). In addition, Richardson et al. found that the transfer of envelope proteins of HIV-infected cells might promote antigen presentation more efficiently and extensively, strengthening antiviral immunity (24). Furthermore, it was reported that the cellular prion protein [PrP(C)], a GPI-anchored protein, is transferred between cells by trogocytosis, which may play an important role in the pathogenesis of prion disease (37). Moreover, Wu et al. showed that the T cell costimulatory molecule CD137 expressed in activated T cells and NK cells, which was induced by the *lmp-1* gene of Epstein-Barr virus (EBV) and the gene *tax* of human T cell virus (HTLV-1), was transferred to APCs by trogocytosis to form the CD137-CD137L complex. Subsequently, the complex was internalized and degraded, which weakened the function of T cell costimulation mediated by CD137 and promoted the immune escape of the virus (68).

The trogocytosis by parasites was reported in unicellular parasites, such as amoebae and *Trypanosoma*, which was beneficial to avoiding attack by host immune defenses and promoting their invasion and killing of host cells (33, 69, 70). Ralston et al. found that *Entamoeba histolytica* (*E. histolytica*) trophozoites use trogocytosis to achieve immune escape and kill host cells by destroying and ingesting host cell membrane fragments to obtain host antigens, which is called amoebic trogocytosis (33). Interestingly, unlike the trogocytosis observed between immune cell-mediated information transmission, amoebic trogocytosis was lethal, which resulted in the loss of cell membrane integrity, degradation of nuclear DNA, and loss of mitochondrial potential of target cells as shown in **Figure 2** (31, 70). In addition, Mukherjee et al. reported that trogocytosis occurred between the epimastigotes of *Trypanosoma cruzi* (69).

Similarly, trogocytosis-mediated immune escape was also observed in tumor progression. Zeng et al. found that the ectopic CD137 expression on Hodgkin and Reed-Sternberg (HRS) cells of Hodgkin's lymphoma (HL) induced by EB virus suppressed immune responses *via* trogocytosis. The ectopically expressed CD137 on HRS cell could bind CD137L and cause an internalization of CD137L on the HRS cells themselves as well as on surrounding APCs, resulting in decreased PBMC proliferation and IFNγ secretion (68, 71). In conclusion, ectopic CD137 expression on HRS cells not only dampened immune activation by reducing CD137L levels on APCs, but also transduced an activation signal into HRS cells leading to the secretion of IL-13 weakening a cellular anti-HL immune responses by deviating immunity toward a Th2 response (71). In addition, CD30 (TNFRSF8) on HRS cells (72), PD-1 (73), and CTLA-4 on T cells were transferred to immune cells or tumor

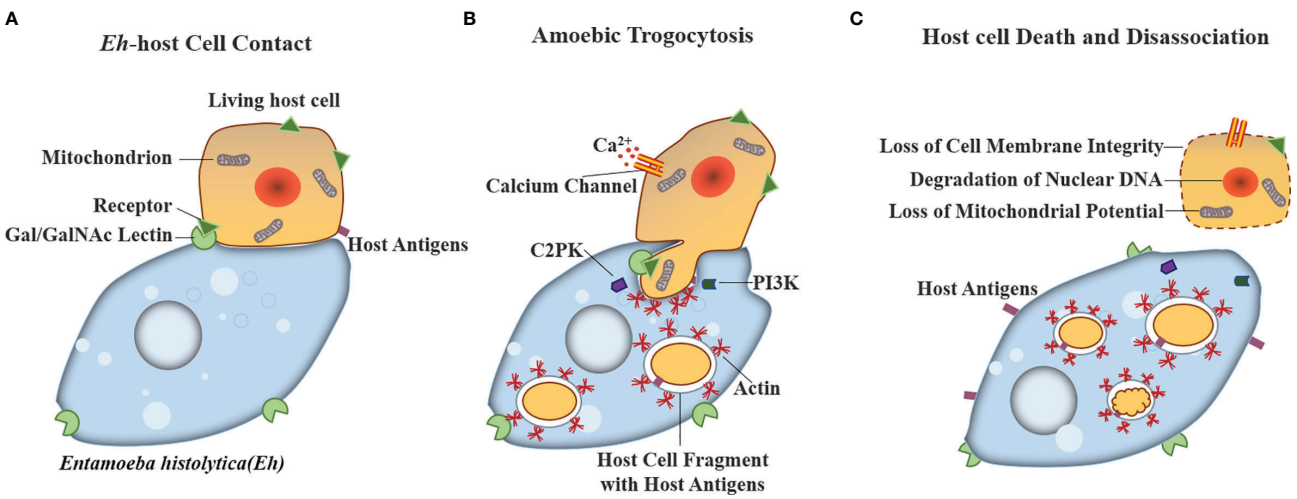

FIGURE 2 | Model for host cell killing *via* amoebic trogocytosis. *Entamoeba histolytica* use trogocytosis to achieve kill host cells by three steps: **(A)** Contact to host glycoproteins containing Gal/GalNAc is mediated by the amoeba surface Gal/GalNAc lectin. **(B)** Amoebic trogocytosis is initiated. The process involves the signal transduction including PI3K and EhC2PK, both of which induce actin polymerization to ingest fragments of host cell material. Host cell intracellular calcium becomes elevated because of the activation of calcium channels. **(C)** Host cell death can be reflected in degradation of nuclear DNA and the loss of membrane integrity and mitochondrial potential.

cells by trogocytosis, giving HRS cells a growth advantage and promoting the immune escape of HL (71).

Attenuating Therapeutic Efficacy in Tumor Therapies

Trogocytosis is also involved in attenuating therapeutic efficacy in tumor therapies. Some studies have revealed that FcγR-mediated trogocytosis by immune cells reduces the effects of a variety of monoclonal antibody (mAb)-based therapies (74–78). Daratumumab, a monoclonal antibody against CD38, was applied to treat multiple myeloma (MM) by inhibiting the growth of tumor cells expressing CD38. Krejcik et al. discovered that monocytes' and granulocytes' gnawing of myeloma cells resulted in the loss of cell membrane fragments containing the daratumumab-CD38 complex, which led to the occurrence of daratumumab resistance during the treatment of MM (78). Similarly, the occurrence of rituximab (RTX) resistance, an anti-CD20 monoclonal antibody widely used in the treatment of non-Hodgkin's lymphoma (77), mantle cell lymphoma (75), and chronic lymphocytic leukemia (76), was attributed to trogocytosis of CD20 on the surface of malignant B cells, which affected the therapeutic effect. Moreover, it was reported that the transfer of P-glycoprotein (functioning as an efflux pump of chemotherapeutics) between cells by trogocytosis led to the multidrug resistance of tumors (74). In addition, Chimeric antigen receptors (CARs) are artificially synthesized receptors that specifically reprogram the functions of T cells and are an effective new treatment for hematologic malignancies (79). However, researchers have found that some tumor cells were antigen-negative or antigen-low condition with unknown mechanism different from complete and permanent antigen loss, which could not be killed by CAR T cells and become a hidden danger that leads to tumor recurrence (80–82). Interestingly, Hamieh et al. revealed that CARs provoked reversible antigen loss through trogocytosis, an active process in which the target antigen is transferred to T cells, thereby decreasing the target density on tumor cells and abating T cell activity by promoting fratricide T cell killing and T cell exhaustion (82).

Hypersensitivity and Autoimmune Diseases

Trogocytosis breaks the immune balance of the body, leading to hypersensitivity reactions and autoimmune diseases, which undoubtedly reflects as a "foe" of trogocytosis endangering human health. Miyake et al. reported that the transfer of large amounts of pMHC II from DCs to basophils by trogocytosis played an essential role in mouse atopic dermatitis by improving antigen presentation and promoting hypersensitivity reactions (52). In addition, Rossi et al. showed that epratuzumab (humanized anti-CD22 antibody), currently in clinical trials for B-cell lymphomas and autoimmune diseases, induced the reduction of multiple B-cell antigen receptor-modulating proteins (CD19, CD21, and CD79b) on the surface of B cells *via* their trogocytosis to effector cells. The reduction of CD19 levels was implicative for the efficacy of epratuzumab in autoimmune diseases because elevated CD19 had been correlated with susceptibility to SLE in animal models as well as in patients. The results suggest that B cells' modulation of key regulatory proteins by trogocytosis may be an important mechanism for immunotherapy with autoimmune diseases (83).

Transplant Rejection and Anti-Rejection

Trogocytosis plays both friend and foe roles in hematopoietic stem cell transplantation. Previous studies reported that the

transfer of intact MHC-I molecules from the surface of the recipient cell to the donor cell by trogocytosis, on the one hand, was beneficial to transplants to avoid surveillance of host immune cells, which reduced rejection in the recipient to achieve successful transplantation (84, 85). On the other hand, allograft rejection is initiated by recipient T cells recognizing donor MHC molecules displayed on graft leukocytes migrating to the host's lymphoid organs (86). Extensive and bidirectional transfer of MHC II molecules between donor and recipient cells *in vivo* was a continual process that through the life of the donor graft, which might involve trogocytosis (87, 88). *In utero* hematopoietic cellular transplantation (IUHCT) has great promise for treating congenital diseases of cellular dysfunction, such as sickle cell disease and immunodeficiency disorders. However, repeated failures in clinical cases of IUHCT in immunodeficiency disease force the establishment of the fetal immune system and prenatal tolerance. It was reported that the surface of tolerant host NK cells during development displayed a low level of donor MHC, and the trogocytosis-mediated transfer of donor MHCs to the recipient was an intrinsic mechanism for the regulation of the development and maintenance of NK cell tolerance in prenatal chimeras (89). The existing studies on trogocytosis involved in transplantation rejection mainly focus on blood system transplantation, while it remains to be explored whether trogocytosis participates in transplantation rejection of parenchymal organs, such as blood vessels, the heart, and kidneys.

In conclusion, trogocytosis is widely involved in various physiological processes and plays a vital role in the immune system and relevant diseases. The results show that trogocytosis is both a friend and a foe of the immune system in different immune environments (**Figure 1**). Trogocytosis-mediated material exchange and signal transmission between immune cells participated in killing *T. vaginalis* and tumor cells, assisted the immune escape of grafts, and promoted resistance to antibody-mediated autoimmune disease. As a foe, trogocytosis accelerated *F. Tularensis* spread among macrophages, took part in *E. histolytica* killing human cells, promoted allergic responses by basophils, assisted immune escape of virus and tumor cells, and aggravated transplant rejection.

REGULATORY MECHANISM OF TROGOCYTOSIS

The characteristic of the mechanism underlying trogocytosis is a key question, although the mechanism of trogocytosis has not been fully demonstrated. Related studies have shown that it is similar to phagocytosis. Trogocytosis was generally a biological process between cells mediated by the formation of the immunological synapse or by the contact of ligands (adhesion molecules, chemokines, antibodies, complements, etc.) and receptors (14, 22, 90–93) with involvement of actin and PI3K (Phosphoinositide 3-kinase) (22, 52, 94). In specific cell types, TC21, RhoG, Src, Syk intracellular calcium and myosin light-chain kinase also played an important role (22, 52, 94). All of these proteins above are also involved in phagocytosis except TC21 (94). When the receptors of trogocytosis-associated cells recognize the corresponding ligands on the target cells, the trogocytosis-associated cells undergo an energy-consuming process, including actin cytoskeletal remodeling and signal transmission and then membrane scission and cell engulfment occur (92, 95, 96). The membrane fragments containing ligands of target cells are captured by trogocytosis-associated cells and then either presented on the surface of trogocytosis-associated cells or internalized, processed, and degraded. Trogocytosis by T, B and NK cells occurs with formation of the immunological synapse (97, 98). CTL acquire membrane fragments with antigenic peptides from target cells through engagement of the T cell receptor (99). Harshyne et al. showed that DC trogocytosis obtained MHC molecules through phagocytic receptors (e.g., scavenger receptors) (7). In addition, FcγR- and complement receptor-mediated trogocytosis by monocytes, macrophages, and neutrophils was mainly involved in killing pathogens and eliminating autoantigens and autoantibodies (39, 100, 101). Sjöström et al. showed that murine NK cells acquired MHC I from surrounding cells by the inhibitory receptor Ly49 family and displayed them at the cell surface. At the same time, the uptake of the inhibitory ligand H-2D^d by NK cells was also dependent on the Ly49A receptor, and blocking the H-2Dd/Ly49A interaction would inhibit the uptake of H-2Dd (102). Another potential mechanism for trogocytosis is the transfer of APC molecules to the T cell *via* microclusters. Some researchers have observed small clusters of MHC being transferred from APC to T cells during the immune synapse (32, 97). Further support for microclusters as a mechanism for trogocytosis, is that microcluster formation is found to be resistant to treatment with PP2 (103).

The results of Aucher et al. showed that the trogocytosis by CD8$^+$ and CD4$^+$ T cells was partially or completely inhibited by inhibitors of cell activation, such as actin polymerization, kinase (such as Src-kinase, Syk-kinase, and PI3K), and low temperature (4°C), while B cells were not, which indicated that the trogocytosis by B cells might not depend on the process of cell activation. This difference was not attributed to the affinity of the B-cell receptor for its cognate antigen being higher than the affinity of the T-cell receptor for its antigen. Rather, it was related to the ability of trogocytosis-associated cells to conjugate with target cells in the presence of inhibitors (92). In addition, it was reported that amoebic trogocytosis could be inhibited by treatment with cytochalasin D, Gal/GalNAc (D-galactose/N-acetyl-D-galactosamine) lectin inhibitor, PI3K inhibitor, amoebic AGC kinase 1 (EhAGCK1) inhibitor or the mutation (96), amoebic cysteine protease (EhCP) inhibitor (104), amoebic C2 domain protein kinase (EhC2PK) inhibitor, or mutation at low temperature (4°C), which led to a reduction in the killing rate of host cells (31). Among them, the AGC kinase family affects actin dynamics by manipulating the downstream PI3K, which influences the action of trogocytosis (**Figure 2**) (33, 105, 106). EhAGCK1 specifically participated in trogocytosis but not phagocytosis of dead cells, while EhAGCK2 participated in all actin-dependent endocytosis processes (96). Inhibition of EhCP

could only block amoebic phagocytosis and could not block amoebic phagocytosis (104). The process of trogocytosis requires the participation of physiological temperature, actin rearrangement, Gal/GalNAc lectins, related enzymes (such as Src-kinase, Syk-kinase, and PI3K), and PI3K signals.

Pham et al. showed that the interruption of actin polymerization and lack of energy would block the process of trogocytosis, and blocking PI3K activity delayed the process of trogocytosis. Alternatively, the inhibition of Src kinases activity slowed the process and reduced the degree of trogocytosis (41). Previous studies have shown that inhibitors of trogocytosis mainly include ATPase inhibitors, actin skeleton blockers, Src, Syk, PIK3 pathway kinase inhibitors, and acidification inhibitors (62, 92, 107). Concanamycin A (an ATPase inhibitor) abrogated Ag-specific trogocytosis (62). Cytochalasin D (actin skeleton blocker) promoted actin depolymerization (41). Wortmannin (PI3K inhibitor) and PP2 (tyrosine-protein kinase Src inhibitor) significantly inhibited the trogocytosis by $CD4^+$ and $CD8^+$ T cells (41, 92). Picetanannol (a tyrosine-protein kinase Syk inhibitor) inhibited the trogocytosis by T cells and neutrophils (4). Ammonium chloride (an acidification inhibitor) reduced amoebic trogocytosis and cell killing but did not weaken initiation while inhibiting the process of receptor dependence (107).

Although the difference of mechanism between trogocytosis and phagocytosis is not yet clear, we have listed some aspects. Trogocytosis requires proteins involved in membrane bending and scission (95, 108) and a small GTPase (94), none of which normally has the role in engulfment and internalization of target cells during phagocytosis. There are many cells being capable of both trogocytosis and phagocytosis, such as neutrophils, macrophages, E. histolytica. How does a cell decide to initiate trogocytosis or phagocytosis? Receptor-ligand interactions may play certain roles. TCR, BCR, KIR, Ly49A receptor, phagocytic receptors, CR3, FcγR, EphA2, EphB and Gal/GalNAc Lectin receptor have been reported to be involved in the trogocytosis of different types of cells (3, 11–13, 15, 21, 27, 32, 40, 48, 62, 66–68, 95, 101, 108). However, whether the receptor is necessary for the initiation of gnawing remains unclear. Further, trogocytosis is regulated by Src and Syk protein kinases and is dependent on ATP, PKC, Ca^{2+}, and actin cytoskeleton. GTPases (TC21, RhoG) and PI3K are also regarded as key factors in trogocytosis and are necessary for the trogocytosis by T cells to obtain MHC molecules from APCs mediated by TCR (**Table 1**) (44, 90, 92, 94). In other words, changes in the cytoskeleton initiate trogocytosis; therefore, the factors that affect the movement of the cytoskeleton can regulate trogocytosis. Finally, the ending of the cell that has been trogocytosed is an almost unexplored field. Why does trogocytosis cause cell death only under certain circumstances? Are additional factors, such as toxins, needed to kill cells through trogocytosis? Since trogocytosis requires direct contact, toxins would need to be highly specific to the cell that is being trogocytosed. How does the trogocytosed cell die post trogocytosis? Is the death of the trogocytosed cell attributed to the activation of the cell death pathway, or the accumulation of physical damage? Conversely, when trogocytosed cells are not killed by trogocytosis, how do they retain cellular integrity? Are cellular repair pathways activated in trogocytosed cells? All the above questions are worthy of our in-depth discussion.

APPLICATIONS OF TROGOCYTOSIS

As a profound demonstration of the function and characteristics of trogocytosis, potential applications of trogocytosis have been proposed. On the one hand, considering the adverse role of trogocytosis in the immune system, such as accelerating *F. Tularensis* spread among macrophages, participating in *E. histolytica* killing human cells, promoting allergic responses by basophils, assisting the immune escape of virus and tumor cells, and aggravating transplant rejection, the inhibition or disturbance of the process of trogocytosis is a potential treatment for some diseases. For example, as trogocytosis is strictly dependent on cell-to-cell contact, disturbing or destroying the process of contact may regulate or block subsequent events, which can provide new ideas for the treatment of diseases. Rossi et al. reported that the anti-CD22/CD20 bispecific antibody could be applied to treat lupus and other autoimmune diseases by reducing the depletion of B cells through enhancing trogocytosis (109). On the other hand, the characteristics and mechanism of trogocytosis can also be applied to many technical fields. Firstly, trogocytosis can be used in the auxiliary diagnosis. For example, due to the critical role of myelin-autoreactive T cells in peripheral blood in multiple sclerosis (MS), the detection of the recognition of T cells on their own APCs by protein transfer analysis can be used to assist in the diagnosis of MS, which is based on the ability of T cells to gnaw membrane proteins from autologous APCs (35). Secondly, trogocytosis can also be applied to find new TCR ligands. Generally, the discovery of TCR ligands has greatly facilitated the identification of disease-specific T cells. However, in many cases, specific ligands have not been defined because of the lack of an applicable method to detect new ligands. As a deep study of trogocytosis, Wang and colleagues used the phenomenon of trogocytosis to develop a cell-based selection platform to discover TCR ligands combined with a peptide-MHC library, which would help in studying the immune mechanisms of diseases and identify new targets for immunotherapies (110). In addition, Daubeuf and Puaux et al. proposed a method based on trogocytosis to capture APC membrane fragments combined with flow cytometry to detect, quantify, characterize and purify antigen-specific lymphocytes. The main advantage of this method is the compatible detection of the phenotypes and functional markers of lymphocytes, which were sorted and used in subsequent experiments or even treatment procedures (111, 112). Furthermore, it would be an exciting concept if trogocytosis could be used to engineer cell behavior, trafficking, and function. For example, CCR7 might be introduced on NK cells to alter their tissue recruitment based on the studies of Marcenaro and Somanchi (39, 42). In conclusion, the potential application of trogocytosis may involve other events and fields based on a deep understanding of the mechanism of trogocytosis in the future.

TABLE 1 | Compared the trogocytosis by different cell types.

Cell type		Involved Biological Aspects	Involved Receptors	Mechanisms (involved molecules)	Transferred Membrane Molecules	Outcomes	References
Immune Cell	**T cell**	To capture APC molecules (pMHC, costimulatory molecules); To obtain the inner membrane protein H-RAS^{G12V} from tumor cells	TCR	Actin, PI3K, Small GTPase (TC21, RhoG), Src, Syk intracellular calcium, myosin light-chain kinase…	pMHC, Costimulatory molecules (CD28, CD54, CD80/CD86), RAS^{G12V}	Regulating T cell proliferative signals and sustaining their activation; Inhibition of the newly arriving T cells and the Ag-experienced T cells; Modulating the effector-cytokine production, differentiation of trog^{+} CD4^{+} T cell and immune response; Promoting the efficiency of T and NK cells killing tumor cells	(5, 15, 16, 22, 32, 48–52, 55–60, 64, 94)
	Treg cell	To deplete pMHCII and CD80/CD86 from APCs;	TCR		pMHC II, CD80/CD86	Reducing the capacity of DCs to present antigen and maintain immune homeostasis; Suppressing T cell immune responses	(3, 17, 61)
	CTL	To acquire membrane fragments with antigenic peptides from target cells; To obtain inhibitory molecules (PD-L1) from APCs	TCR		PD-L1	Regulating the immune response and recycle the molecule	(62, 99)
Immune Cell	**B cell**	To extract and present antigen tethered tightly to a non-internalizable surface; To remove epitopes from red blood cells; To obtain the avian flu receptor of α2,3 sialic acids from monocytes	BCR	Actin, PI3K, , Small GTPase (TC21, RhoG), Src, Syk intracellular calcium, myosin light-chain kinase…	Antigen tethered tightly to a cell surface, Epitopes of red blood cells, Avian flu receptor (α2,3 sialic acids)	Initiating the humoral immune response; Mediating the inhibitory effect on the immune response; to increase their susceptibility to H5N1	(27, 63, 67)
	NK	To extract CCR7 from CCR7^{+} cells; To obtain the inner membrane protein H-RAS^{G12V} from tumor cells; To obtain and display MHC I	KIR, The inhibitory receptor Ly49 family (Ly49A receptor)		CCR7, H-RAS^{G12V}, MHC I, H-2Dd	Migrating to the site of killing mature DCs and T lymphoblasts with the help of chemokines CCL19/CCL21; Promoting the secretion of INF-γ and TNF-α, the proliferation lymphocyte, and the efficiency of NK cells killing tumor cells; Preventing the NK cell-mediated killing of normal cells	(39, 40, 42, 64, 102)
	DC	To obtain MHC molecules	Phagocytic receptors (e.g., scavenger receptors)		MHC molecules, CD137	Antigen presentation	(7, 68)
Immune Cell	**Mø**	To remove antigens from target cells, such as T cells and NK cells; To participate in the death of antibody-opsonized tumor cells; Transfer of *F. tularensis* and *S. enterica* among macrophages	CR3 FcγR	Actin, PI3K, Small GTPase (TC21, RhoG), Src, Syk intracellular calcium, myosin light-chain kinase…	Antigens, Intracellular pathogens (*F. tularensis* and *S. enterica*), CD137	Affecting the function of target cells but not obtain new proteins and new functions; Killing tumor cells; Accelerating the spread of intracellular bacteria	(18, 26, 38, 41, 66, 68)
	Neutrophil	To nibble and remove membrane fragments from *Tv*; To participate in the destruction of antibody-opsonized cancer cells	CR3 FcγR		Membrane fragments of *Tv* or cancer cell	Killing pathogens and tumor cells	(4, 11–13)
	Monocyte	To remove well-exposed antibody-gp120 complexes from the surface of infected cells	CR3 FcγR		Antibody-gp120 complexes	Resulting in immune escape of HIV; Killing pathogens	(21, 39, 100, 101)
E. histolytica		To destroy and ingest host cell membrane fragments to obtain host antigens	Gal/GalNAc Lectin receptor	Actin, PI3K, Src, Syk, Gal/GalNAc lectin, EhAGCK1, EhAGCK2, EhCP, EhC2PK	Host antigens	Achieving immune escape and killing host cells	(31, 70)

T, T lymphocyte; B, B lymphocyte; APC, antigen presenting cell; Treg, regulatory T cell, CTL, cytotoxic lymphocyte; NK, natural killer cell; DC, dendritic cell; Mø, macrophage; pMHC, antigenic peptide-MHC complex; TCR, T cell receptor; BCR, B cell receptor; Tv, Trichomonas vaginalis. KIR, killer Ig-like receptors; CR3, complement receptor 3; FcγR, Fc gamma receptors; PI3K, phosphoinositide 3-kinase; Src, Src kinase family; Syk, Syk kinase family; HIV, human immunodeficiency virus; PGC, primordial germ cells; EphA2, Eph receptor A2; EphB, Eph receptor B; Gulp1, PTB domain-containing engulfment adapter protein 1; RacGEF, Rac-specific guanine nucleotide exchange factor; Tiam2, TIAM Rac1 Associated GEF 2; Gal/GalNAc, D-galactose/N-acetyl-D-galactosamine; EhAGCK1, amoebic AGC kinase 1; EhAGCK2, amoebic AGC kinase 2; EhCP, amoebic cysteine protease; EhC2PK, amoebic C2 domain protein kinase.

CONCLUSION AND PERSPECTIVES

In summary, trogocytosis plays a beneficial or adverse role in the immune system in different environments. On the one hand, trogocytosis strengthens immune defenses (antigen presentation and immune cell activation) and is accompanied by an increase in the probability of pathological immune damage. On the other hand, pathogens and abnormal cells also use trogocytosis to evade human immune surveillance. Based on existing research, it is impossible to generalize whether trogocytosis in the immune system is a foe or a friend because immunity is a double-edged sword. Trogocytosis can be applied to find therapeutic targets in infectious diseases, tumors and other diseases. However, given the negative role of trogocytosis in the immune system, disrupting its process of trogocytosis may be applied to treat the immune escape of tumors and pathogens, hypersensitivity, autoimmune diseases and graft-versus-host disease. At present, research on trogocytosis has not fully elucidated its mechanism and is mainly limited to the cytoskeleton's role. Trogocytosis needs to be studied and clarified in depth.

There are several questions worth considering in future research. First, what are the different mechanisms by which trogocytosis regulates similar processes under different conditions, leading to different functional consequences? Second, because many cells are capable of trogocytosis and phagocytosis, such as *E. histolytica*, neutrophils, and macrophages, how does the cell decide to initiate trogocytosis or phagocytosis? Is it receptor-ligand binding or other unknown and more direct ways? Third, the proteins on the cell surface rely on hydrophobic interactions to exist stably on the cell membrane, so the transfer of proteins between cells needs to destroy or overcome this hydrophobic effect. The force required to pull out the intact membrane protein from the hydrophobic lipid bilayer as proven to be similar to the force required to break high-affinity protein interactions (e.g., antigen-antibody interactions) as early as 1978 (113), so how do trogocytosis-positive cells produce such a huge pulling force to "tear" membrane fragments and membrane proteins from the target cell? Finally, the problem of cell membrane repair post trogocytosis is also crucial. Are cellular repair pathways activated in trogocytosed cells? Specifically, since calcium influx is a trigger for cell membrane damage repair (114), does calcium influx occur in trogocytosed cells? As far as the result of trogocytosis is concerned, the rearrangement and combination of functions of cells through trogocytosis makes cells functions more diverse. Of course, trogocytosis somewhat improves the efficiency of organisms and reduces costs.

AUTHOR CONTRIBUTIONS

JS advocated writing this review. SZ collected literature and wrote the manuscript. LZ, SX, and YH collected literature. ZW provided some suggestions for this review. JS and ZW reviewed, edited and approved its final version. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 81802036 and 81871682), the National Research and Development Plan of China (No. 2020YFC1200100 and No. 2016YFC1200500), the Natural Science Foundation of Guangdong Province of China (2020A1515010896), the China Postdoctoral Science Foundation (No. 2018M631027 and 2019T120770).

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

frontiers | Frontiers in Immunology

REVIEW
published: 07 April 2022
doi: 10.3389/fimmu.2022.865395

The Role of MIF and IL-10 as Molecular Yin-Yang in the Modulation of the Host Immune Microenvironment During Infections: African Trypanosome Infections as a Paradigm

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Edited by:
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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: 29 January 2022
Accepted: 14 March 2022
Published: 07 April 2022

Citation:
Stijlemans B, Schoovaerts M, De Baetselier P, Magez S and De Trez C (2022) The Role of MIF and IL-10 as Molecular Yin-Yang in the Modulation of the Host Immune Microenvironment During Infections: African Trypanosome Infections as a Paradigm. Front. Immunol. 13:865395. doi: 10.3389/fimmu.2022.865395

African trypanosomes are extracellular flagellated unicellular protozoan parasites transmitted by tsetse flies and causing Sleeping Sickness disease in humans and *Nagana* disease in cattle and other livestock. These diseases are usually characterized by the development of a fatal chronic inflammatory disease if left untreated. During African trypanosome infection and many other infectious diseases, the immune response is mediating a see-saw balance between effective/protective immunity and excessive infection-induced inflammation that can cause collateral tissue damage. African trypanosomes are known to trigger a strong type I pro-inflammatory response, which contributes to peak parasitaemia control, but this can culminate into the development of immunopathologies, such as anaemia and liver injury, if not tightly controlled. In this context, the macrophage migration inhibitory factor (MIF) and the interleukin-10 (IL-10) cytokines may operate as a molecular "Yin-Yang" in the modulation of the host immune microenvironment during African trypanosome infection, and possibly other infectious diseases. MIF is a pleiotropic pro-inflammatory cytokine and critical upstream mediator of immune and inflammatory responses, associated with exaggerated inflammation and immunopathology. For example, it plays a crucial role in the pro-inflammatory response against African trypanosomes and other pathogens, thereby promoting the development of immunopathologies. On the other hand, IL-10 is an anti-inflammatory cytokine, acting as a master regulator of inflammation during both African trypanosomiasis and other diseases. IL-10 is crucial to counteract the strong MIF-induced pro-inflammatory response, leading to pathology control. Hence, novel strategies capable of blocking MIF and/or promoting IL-10 receptor signaling pathways, could potentially be used as therapy to counteract immunopathology development during African trypanosome infection, as well as during other infectious conditions. Together, this review aims at

summarizing the current knowledge on the opposite immunopathological molecular "Yin-Yang" switch roles of MIF and IL-10 in the modulation of the host immune microenvironment during infection, and more particularly during African trypanosomiasis as a paradigm.

Keywords: MIF, IL-10, glucocorticoids, African trypanosomiasis, *T. brucei*, *T. congolense*

INTRODUCTION

Trypanosomes represent a group of unicellular protozoan parasites from the genus *Trypanosoma* belonging to the order of the Kinetoplastida. These parasites are the causative agents of trypanosomiasis, a set of debilitating human and veterinarian diseases with huge medical but also socio-economical implications. The genus Trypanosoma contains about 20 different species of trypanosomes that cause infections in a large variety of vertebrates (1). Yet, only two sub-species of the extracellular *Trypanosoma brucei* (*T. brucei*) parasite have been shown to establish significant diseases in humans, causing Human African Trypanosomiasis (HAT), also known as Sleeping Sickness disease, endemic in 36 countries in Sub-Saharan Africa. *Trypanosoma cruzi* (*T. cruzi*) is an obligate intracellular parasite that causes Chagas' disease, a debilitating infection mostly widespread in Latin-America. Both diseases are part of the Neglected Tropical Diseases (NTDs), which are mostly affecting the world's poorest and less developed populations (1, 2).

African trypanosomes are a group of extracellular parasites found in Sub-Saharan Africa, including both human-infective species as well as species only infecting vertebrate animals. These flagellated unicellular parasites are transmitted through the bite of blood-feeding tsetse flies from the *Glossina* species, during which the parasites are injected in the mammalian circulation. These parasites are responsible for HAT in humans and Animal African Trypanosomosis (AAT), also known as *Nagana* disease, in cattle and other livestock (3, 4). The African trypanosome species *Trypanosoma brucei* can be subdivided into three subspecies, from which two cause infections in humans. *Trypanosoma brucei gambiense (T. b. gambiense)*, present in West and Central Africa, causes a chronic form of HAT representing approximately 98% of the cases. In East and Southern Africa, a more acute form of the disease is caused by *Trypanosoma brucei rhodesiense (T. b. rhodesiense)*, a parasite for which animals represent the main reservoir, but which can infect humans too (1). HAT is characterized by two main disease stages. First, after transmission through the bite of the tsetse vector, parasites mainly spread to and proliferate in the lymphatics and the blood system of the host, triggering the haemolymphatic stage of the disease. Recurrent complications are anaemia, fever, lymphadenopathy and liver pathology (1, 5, 6). The second stage of infection, called meningo-encephalitic phase, occurs typically weeks after *T. b. rhodesiense* and months after *T. b. gambiense* infection, when parasites invade the central nervous system (CNS), first by migrating to the circumventricular organs (CVOs) and peripheral ganglia which have fenestrated vessels, followed by crossing the blood-brain barrier (BBB) and the blood-CSF (cerebrospinal fluid) barrier, resulting in neurological complications and death if left untreated (6–9). *Trypanosoma brucei brucei (T. b. brucei)*, the third subspecies of *T. brucei*, causes similar pathological features as the two other forms and is therefore used as a model parasite, yet it can only cause AAT (10). *Trypanosoma congolense (T. congolense)* and *Trypanosoma vivax* (*T. vivax*) are considered the most important causative agents of AAT. In contrast to *T. brucei*, these species were shown to be strictly intravascular, without a migration to extravascular places within the host. Additionally, *Trypanosoma evansi* (*T. evansi*), the causative agent of Surra, can also cause diseases in animals but in contrast to the other trypanosomes can be transmitted mechanically, which favor its worldwide distribution compared to other species (11). The most common immunopathology and major cause of death linked to *Nagana* disease is thought to be anaemia, which can however be accompanied by other complications such as fever, weight loss and liver pathology (12).

Due to millions of years of co-evolution with their mammalian host, African trypanosomes have developed several mechanisms enabling them to escape the host immune system. Indeed, already at the inoculation stage, immunomodulatory components from tsetse saliva can suppress host immune response (13), thereby allowing early parasite establishment. Furthermore, to reduce inflammation, trypanosomes release early factors and vesicles containing factors, which upregulate the IL-10 production and prevents TNF expression (14, 15). For instance, a kinesin heavy chain released by *T. brucei* (TbKHC1) was shown to trigger SIGN-R1 receptor-dependent induction of IL-10 production in myeloid cells, resulting in arginase-1 activation and increased polyamine production. This promotes early trypanosome growth and favors parasite settlement in the host, concomitant with reduction of nitric oxide production (16, 17). Bioinformatics analysis revealed the presence of TbKHC1 gene homologs in other trypanosomes. In addition, a recent study indicates that *T. b. brucei* and TbEVs (extracellular vesicles) seem to display opposite but complementary effects in the host, establishing a balance between parasite growth and controlled immune response, at least during the early phase of infection (15). Later on, also other immune evasion mechanisms such as antigenic variation of the variant-specific surface glycoprotein (VSG) coat, clearance of surface bound antibodies, polyclonal lymphocyte activation and finally, the shutdown of their "main enemy" within the immune system, namely the B lymphocytes play a key role in parasite survival (18–28). These mechanisms are the main reason for the establishment of a chronic African trypanosomiasis disease and

the lack of a protective vaccine so far. This, together with the fact that some trypanosome strains developed resistance to several treatments, and that the control or eradication of tsetse flies remains very challenging in many remote areas, explains why increasing efforts have been put in understanding the mechanisms underlying the modulation of the host immune microenvironment caused by the infection (29). Furthermore, new strategies capable of reducing African trypanosome infection-associated immunopathologies, might be an alternative approach to target this parasitic infection with unmet medical need.

It is now generally considered that parasitaemia control is greatly dependent on parasite-mediated quorum sensing whereby cell-cycle arrested, quiescent, stumpy forms differentiate from proliferative slender forms that may escape immune clearance (30), and host-dependent antibody-mediated killing (31). Specific antibodies are raised against the immunodominant VSG of the parasite population, through both T-cell-dependent and T-cell-independent B cell responses (32–34). Next to the humoral anti-parasite B cell responses, a strong type I pro-inflammatory cellular response is triggered upon African trypanosome infection. Using experimental mouse models of infection, studies identified the production of the pro-inflammatory cytokine IFNγ by NK, NKT and T_H1 cells (35–37), together with the release of several parasite-derived components, as major driving force in the activation of myeloid cells. After this, the macrophages and dendritic cells (DCs) typically display a "classical" or pro-inflammatory activation profile, producing several trypanocidal molecules such as reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), and tumor necrosis factor (TNF) (38–41). Some of these DCs are also referred as TNF/inducible nitric oxide synthase (iNOS)-producing dendritic cells (Tip-DCs) (42, 43). However, if persistent, this early beneficial type I pro-inflammatory response which is essential for parasitaemia control can culminate into the development of immunopathologies, such as anaemia and liver injury (35, 44). Therefore, this pro-inflammatory environment must be controlled by the production of regulatory cytokines such as interleukin-10 (IL-10), TGF-β and IL-27 (42, 43, 45–47). Particularly, the production of IL-10 was shown to be crucial to prevent the development of an uncontrolled inflammation syndrome, associated with early mortality (42, 45). Depending on the trypanosome species and the stage of infection, different cellular sources could contribute to IL-10 production such as NK cells, $CD8^+$ T cells and $CD4^+$ T cells around peak parasitaemia as well as B cells and plasma cells, myeloid cells and hepatocytes as the infection progresses (45, 48–51). Furthermore, the level of IL-10 induction might also differ between different mouse strains (52).

As mentioned earlier, anaemia is considered the most prominent immunopathological complication and main cause of death, linked to AAT. Therefore, for cattle, the concept of trypanotolerance has been described as the capacity of an animal to control anaemia together with the ability to lower parasitaemia (53, 54). Of note, in this review, we will refer to trypanotolerance in mice as the ability to control pathology (e.g. anaemia) which can be monitored, while parasitaemia control is mainly a reflection of circulating parasites within the blood and does not provide information regarding the parasite load in every organ during infection. Thus, this implies the development of a subtle balance between pro- and anti-inflammatory signals capable of limiting parasitaemia while avoiding excessive host tissue damage and death. Some trypanosome species, such as *T. congolense*, have been shown to establish chronic infections in some trypanotolerant cattle species (*e.g.* N'Dama, *Bos taurus*). In these animals, after an early pro-inflammatory response, the immune system can switch the macrophage and DC activation status from classically activated (pro-inflammatory) to alternatively activated (anti-inflammatory) (55, 56). This drives the immune response from a pro-inflammatory type I to a more anti-inflammatory profile, associated with the secretion of characteristic cytokines such as IL-10, IL-27 and TGF-β (42, 45–47).

Although most trypanosomes cannot be considered natural rodent pathogens, experimental mouse models have proven to be very valuable tools to study parasite-host interactions and the modulation of the host immune microenvironment during infection (57). Two different models using the C57BL/6 mouse strain have been studied extensively. For example, infection of C57BL/6 mice with the *T. b. brucei* parasite, *i.e.* pleomorphic AnTat1.1E model, mimics a more "trypanosusceptible" infection model as these mice succumbed earlier from immunopathologies, whereas infection of C57BL/6 mice with *T. congolense, i.e.* Tc13 model, is more considered as a "trypanotolerant" model with establishment of a more chronic infection and longer survival (approximately 1 month versus 3-4 months, respectively). This is linked to the ability of *T. congolense*-infected mice to switch from a type I pro-inflammatory response to an anti-inflammatory response involving IL-10 production (**Figure 1A**). Conversely, *T. b. brucei*-infected C57BL/6 mice exhibit a persistent type I pro-inflammatory response and show more severe anaemia development as compared to *T. congolense*-infected mice (60, 61). Of note, the genetic background of the mice was also found to contribute to susceptibility or tolerance as far as anaemia is concerned, whereby during *T. brucei* and *T. congolense* infection C57BL/6 mice exhibit severe anaemia (yet low parasitaemia) while BALB/c mice exhibit greatly reduced anaemia (yet higher parasitaemia) (62, 63).

Therefore, identification of molecules contributing to persistence of the pro-inflammatory immune response might represent potential therapeutic targets. Increasing evidence has put forward macrophage migration inhibitory factor (MIF) as a key driver (*i.e.* upstream regulator) in the early pro-inflammatory response against African trypanosomes (58, 64, 65), as well as in several inflammatory diseases (66, 67). In addition, in the context of African trypanosome infections, MIF was shown to promote the most important immunopathologies linked to the infection, such as anaemia and liver damage (58, 65). In contrast, IL-10 is considered as key mediator in the anti-inflammatory response needed to counterbalance the pro-inflammatory environment and protect the host from severe

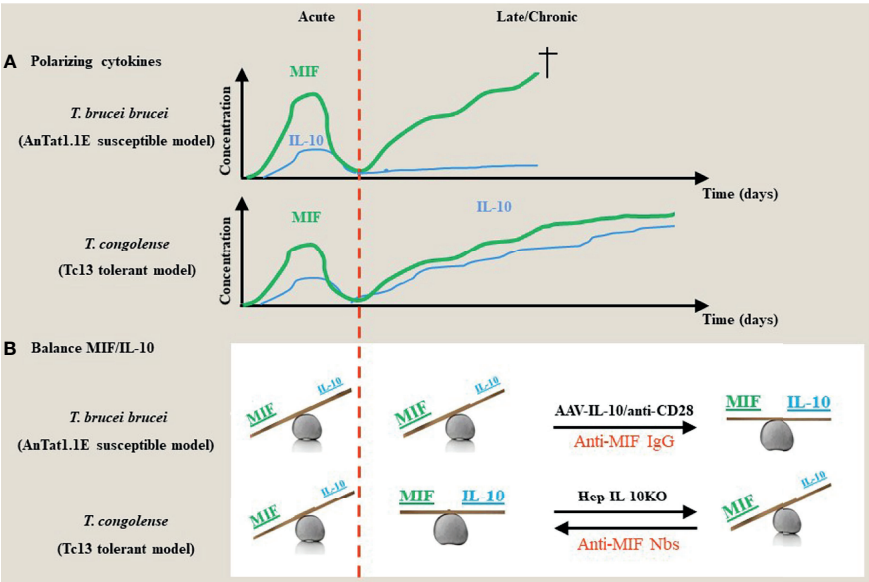

FIGURE 1 | Proposed model for the contribution of the dyad MIF and IL-10 during the acute/chronic stage of a trypanosome infection. **(A)** During the early stages of African trypanosome infection, both in trypanosusceptible and tolerant animals there is induction of MIF, which drives a strong pro-inflammatory response. This is paralleled by an early IL-10 production to prevent excessive tissue damage. During the chronic/later stage of infection in both models, there is a second progressive increase in MIF that further fuels pro-inflammatory cytokines, which contributes to tissue damage and death. However, in contrast to trypanosusceptible animals, trypanotolerant animals are able to mount a second more progressive increase in IL-10 to dampen the pathology-promoting effects of MIF. **(B)** During the early stage of infection, both in trypanosusceptible and tolerant animals, the balance between the polarizing MIF and IL-10 cytokines is tilted towards MIF. During the late/chronic stage, the balance remains in favor of MIF in trypanosusceptible animals, whereas there is a balance between MIF and IL-10 activities resulting in an equilibrium between parasite control and pathology control in trypanotolerant animals. Absence of hepatocyte-IL-10 at the later stages of infection in the trypanotolerant animals alleviates the protective effect and tilts the balance again in favor of MIF and uncontrolled pathology. Strategies that either promote IL-10 production (i.e. AAV-IL-10 or anti-CD28 treatment) or block MIF (such as antibodies or small molecules) establish a new balance that prevents tissue injury (42, 48, 58, 59).

tissue damages, usually leading to death (45, 68, 69). Therefore, this review aims at summarizing the current knowledge on the roles of MIF and IL-10, which can be considered as a molecular "Yin-Yang" in the modulation of the host immune microenvironment during infection, and particularly during African trypanosomosis.

MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF)

Genetics, Receptor Signaling, and Functions of MIF

The macrophage migration inhibitory factor (MIF) protein is an important pleiotropic inflammatory cytokine with chemokine-like functions that has gained a lot of interest since its first functional description approximately 55 years ago. MIF was initially identified as an inhibitor of random macrophage migration, a function that gave this molecule its name (70, 71). However, further information on other biological activities of MIF remained unclear till the first successful cloning of human MIF cDNA in 1989 (72). The human *MIF* gene is located on chromosome 22 (22q11.2) and encodes a highly conserved protein of 115 amino acids with a molecular weight of approximately 12,5 kDa (72). Crystallographic studies revealed that MIF monomers can assemble to form a functional homotrimer (73). The murine *MIF* gene maps to chromosome 10 and shows a similar protein structure compared to the human counterpart (74). Furthermore, all mammalian MIFs (human, mouse, rat and cattle) show approximately 90% amino acid sequence identity (66, 75).

Historically, T cells were considered the main cellular source of MIF (70, 71). However, it is now clear that MIF is produced by a large variety of immune cells including T cells, monocytes, macrophages, DCs, polymorphonuclear cells (PMNs) and B cells (76–81). Additionally, MIF is expressed by non-immune tissues such as several organs of the endocrine system (hypothalamus, pituitary and adrenal glands) and others forming barriers with the environment, including lung, epithelium of the skin and gastrointestinal tract (82, 83). In contrast to most other cytokines, MIF is expressed constitutively and stored in intracellular pools. MIF can then be directly secreted in response to various stimuli such as infection and cytokine stimulation, which if not properly controlled can cause cell and tissue injury (66, 84).

Once secreted, MIF functions as an "early response" cytokine stimulating the pro-inflammatory response. MIF binds to its cognate receptor which consists of a two-component signaling complex. This complex contains the CD74 ligand-binding protein and the CD44 signal transducer (85, 86). In fact, CD74

is the MHC-II invariant chain (li) playing a role in MHC-II peptide loading in antigen presenting cells but on the cell surface it functions as a cognate receptor for MIF. However, CD74 surface expression is not strictly MHC-II dependent as it is also expressed on several MHC-II negative cell types, including endothelial and epithelial cells (87, 88). After MIF binding to the CD74 homotrimer, it recruits and forms a complex with CD44. Both parts are then phosphorylated at their cytosolic domain, triggering the intracellular downstream signaling through activation of Src kinases (86). This results in sustained activation of the extracellular-signal-regulated kinase 1/2 (ERK1/2)/mitogen-activated protein kinase (MAPK) pathway and subsequent activation of cytoplasmic phospholipase A2 ($cPLA_2$). This cascade finally results in a prolonged cell survival and classical/pro-inflammatory activation of myeloid cells. MIF is for example capable of alleviating the inhibition of pro-inflammatory cytokine expression caused by glucocorticoids, thereby turning the myeloid cells into pro-inflammatory cytokine secreting cells (89–91). It was also shown that MIF sustains inflammation by suppressing p53-dependent apoptosis of inflammatory cells (92). Furthermore, other signaling pathways including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), protein kinase B (PKB, also called Akt) and phosphatidylinositol 3-kinase (PI3K) pathways can be activated by MIF in a variety of cells, leading to further activation and pro-survival signals (93). Next to the cognate receptor CD74-CD44, MIF can also interact with the chemokine receptors CXCR2 and CXCR4. Indeed, both were found to associate with CD74 at the cell surface, thereby mediating interaction with MIF. Through these interactions, MIF mediates myeloid cell and T cell recruitment at the site of inflammation, and this also results in the eponymous function of macrophage retention at inflammatory sites (94). In addition, MIF was shown to interact with CXCR7 on B cells. This chemokine receptor forms complexes with CD74 and triggers B cell recruitment and activation upon binding to MIF (95).

The above-described MIF protein is now often referred to MIF-1, due to the existence of a MIF homolog called *D*-dopachrome tautomerase (D-DT) or MIF-2. D-DT shows 34% sequence identity with MIF and a nearly identical 3D structure (96). Functionally, D-DT resembles MIF in its ability to signal through the CD74/CD44 receptor complex, thereby promoting inflammation through for example ERK1/2 MAPK signaling (97). However, D-DT does not have the ability to interact with CXCR2, CXCR4 and CXCR7 and is therefore not capable of mediating the chemokine-like functions of MIF (94, 98). So far, the role of D-DT has not been thoroughly studied in parasitic infections.

Role of MIF in African Trypanosome Infection

African trypanosomes trigger a strong, early type I pro-inflammatory response in the mammalian host, which is however leading to the development of several immunopathologies if persistent (35, 38–41, 44). As described above, the MIF cytokine is a potent inducer of inflammation and involved for example in myeloid cell recruitment and the differentiation of these cells into classically activated M1 cells producing pro-inflammatory cytokines such as TNF (89–94). Therefore, the potential role of MIF in the development of African trypanosome infection-associated pathogenicity was scrutinized in both the *T. brucei* and *T. congolense* models.

During infection with *T. brucei*, both *Mif* gene expression and MIF protein levels are upregulated during the acute phase of infection, whereafter both decline again following clearance of the first peak of parasitaemia (typically day 5-6 post infection) (58). These observations are in accordance with another study that showed increased mRNA levels of MIF in spleen macrophages of *T. b. brucei*-infected and *T. b. gambiense*-infected rats collected at day 4 post infection (p.i.) (99). This is followed by a second more progressive increase when the infection evolves to the later/chronic stage (day 10 post infection and ongoing) (58). Using both MIF-deficient ($Mif^{-/-}$) mice and a neutralizing anti-MIF IgG treatment it was shown that MIF deficiency does not affect the development of parasitaemia (58). However, MIF absence was shown to slightly increase survival of *T. b. brucei*-infected mice. This was linked to reduced serum concentrations of the pro-inflammatory cytokines (IFNɣ, TNF, and IL-6) and an increase in the IL-10 serum concentration, especially during the chronic stage of infection (58).

The strong type I pro-inflammatory response observed in C57BL/6 mice leads to the development of immunopathologies, such as liver injury and anaemia, if persistent (35, 42, 44). Interestingly, *T. b. brucei*-infected $Mif^{-/-}$ mice were shown to suffer from significantly less liver damage compared to WT infected mice (58). This coincided with less infiltration of $CD11b^+$ $Ly6c^+$ myeloid cells, comprising both $CD11b^+$ $Ly6c^{hi}$ $Ly6G^-$ inflammatory monocytes and $CD11b^+$ $Ly6c^{int}$ $Ly6G^+$ neutrophils, which is linked to a reduced expression of the inflammatory monocyte chemoattractant CCL2 and of the neutrophil chemoattractants CXCL1 and CXCL5 in livers of $Mif^{-/-}$ mice compared to infected WT mice (58).

Next to liver pathology, the strong pro-inflammatory immune response elicited during *T. b. brucei* infection culminates in the development of anaemia (35). Using $Mif^{-/-}$ mice as well as anti-MIF IgG treatment, it was shown that MIF deficiency results in a less severe anaemia profile during *T. b. brucei* infection. This correlated with a reduced inflammatory immune response and a restored iron homeostasis in $Mif^{-/-}$ mice leading to an improved erythropoiesis (58, 100). Next to an improved erythropoiesis, the alleviation of anaemia was also found to be linked to the reduced clearance of red blood cells (RBCs) observed in *T. b. brucei*-infected $Mif^{-/-}$ mice in the chronic phase of infection (58). This could in turn be the result of the reduced recruitment and activation of myeloid cells observed in $Mif^{-/-}$ mice.

Next, in 2016, the role of MIF was investigated in the trypanotolerant *T. congolense* model which is a more relevant model for bovine trypanosomosis (60, 61). Similar as for the *T. b. brucei* infection, *T. congolense*-infected $Mif^{-/-}$ mice show no differences in parasitaemia as compared to WT infected mice.

However, MIF absence leads to a more significant increase in median survival time in *T. congolense*-infected mice (65). This is associated with reduced pathogenicity, typically in the chronic phase of infection (approximately 3 months p.i.). Indeed, compared to WT mice, *T. congolense*-infected *Mif*$^{-/-}$ mice showed reduced liver damage, together with reduced hepatosplenomegaly. Furthermore, in the absence of MIF, both chemokines (CXCL1, CCL2) and pro-inflammatory cytokines (IFNγ, TNF, IL-6) known to play a role in pathology of African trypanosome infection are downregulated in the chronic phase of *T. congolense* infection, similar to what happens in the trypanosusceptible model (58, 65). This also coincided with lower infiltration of myeloid cells in the liver of *T. congolense*-infected *Mif*$^{-/-}$ mice compared to infected WT mice (65).

In contrast to *T. b. brucei* infections, the development of anaemia during the chronic phase of *T. congolense* infection seems not to be solely caused by the MIF-dependent pro-inflammatory response, but also results from an additional mechanism, namely hemodilution, which is due to an increased plasma volume (PV) and blood volume rather than by a reduction in red blood cell (RBC) mass (65). This mechanism is dependent on MIF, as *T. congolense*-infected *Mif*$^{-/-}$ mice show reduced hemodilution compared to infected WT mice (65). Next to general anaemia, the hemodilution also leads to several other effects, including thrombocytopenia with impaired coagulation and uncontrolled bleeding as a result (65).

Altogether, these results indicate that MIF is a critical mediator of inflammation and African trypanosome-associated immunopathologies, but not in parasite control. At first glance the observation that MIF is not involved in the control of parasitaemia appears contradictory with the documented contribution of inflammatory cytokines in parasite control and will be discussed in the conclusion section. Anyway, novel therapeutic strategies targeting MIF and/or MIF signaling could constitute a new approach/avenue to limit/tackle these trypanosome-elicited pathologies. In the context of HAT, a recent study conducted on Guinean HAT patients suggested that MIF expression is also increased during infection and coincides with pathology (101). However, it seemed that it does not correlate with the disease stage. Indeed, although MIF expression was found drastically increased in *T. b. gambiense*-infected individuals, this was both the case for HAT patients with active disease as for patients with latent infections (101). Furthermore, investigations on *Mif* gene polymorphisms suggested no correlation between increased MIF polymorphisms and a risk of developing active HAT (102). Thus, results from experimental African trypanosome infection models suggest MIF is correlated with pathology and is a bad prognostic marker, while in HAT it seems that MIF correlates with pathology without prognostic value.

Role of MIF in Other Infections

Trypanosoma cruzi (*T. cruzi*) is the causative agent of Chagas' disease mostly affecting people in Latin-America and main contributor of pathogen-mediated-cardiomyopathy (1). This intracellular parasite can infect and live inside different cell types, in contrast to African trypanosomes which are strictly extracellular. Macrophages are one example of cells that can be infected by *T. cruzi* and play an important role in dissemination of the parasite to other sites within the body. *T. cruzi* parasites replicate inside the cells, whereafter they differentiate back to bloodstream stage parasites after cell burst (1). The host immune response against *T. cruzi* consists of a strong innate immune response with the activation of macrophages leading to NO production. This strong innate response is promoted by the establishment of robust antigen-specific T_H1 $CD4^+$ T cell and cytotoxic $CD8^+$ T cell responses (103). It has been demonstrated that MIF plays a critical protective role during acute *T. cruzi* infection, by inducing the production of several pro-inflammatory cytokines, including IL-12, IL-18, TNF, IL-1β, and IFNγ, during the early phase of infection. Indeed, in contrast to African trypanosome infection, infected *Mif*$^{-/-}$ mice were highly susceptible to *T. cruzi* infection, due to impaired pro-inflammatory cytokine production (104). MIF was also found to directly promote *T. cruzi* killing by macrophages and potentiate the effect of interferon-gamma on *T. cruzi* killing by peritoneal macrophages due to its ability to promote endogenous production of TNF, NO and ROS, as well as their trypanostatic/trypanocidal actions (105). MIF has also been linked to the development of immunopathologies in chronic *T. cruzi* infections. The main clinical symptoms associated with chronic *T. cruzi* infections are heart inflammation and dysfunction, also known as chronic chagasic cardiomyopathy (CCC). MIF was overexpressed in the hearts of chronically infected mice, and especially those with high heart inflammation. Similarly, the serum concentrations of MIF in CCC human patients were found significantly increased compared to asymptomatic *T. cruzi*-infected and uninfected humans (106).

Leishmaniasis is one of the most important Neglected Tropical Diseases (NTDs) next to HAT and is caused by protozoan parasites of the genus *Leishmania*. In the mammalian host, these parasites are obligatory intracellular parasites infecting macrophages and other myeloid cell types (107). Depending on the parasite species and the ability of the host to mount an effective immune response, the disease translates itself into different clinical complications including benign ulcers, cutaneous lesions, and systemic visceral complications (107). For example, infection with *Leishmania major* (*L. major*) is known to mainly cause self-healing cutaneous lesions. Parasite control requires a strong type I pro-inflammatory response with the occurrence of classical activation of macrophages and Tip-DCs differentiation, leading to the production of TNF, RNI and ROS and the subsequent killing of intracellular leishmania parasites (64, 108, 109). Several studies reported a protective role of MIF in leishmaniasis. For example, purified recombinant MIF was found to activate murine macrophages to kill *L. major* parasites, with maximal effects at concentrations corresponding to levels found *in vivo* during infection. This protective property of MIF was shown to be dependent on RNIs and endogenous TNF produced by macrophages (110). Consistent with these results, another

study demonstrated that *Mif*$^{-/-}$ mice were highly susceptible to *L. major* infection, resulting in larger skin lesions and a higher parasite load compared to WT mice (111). A study conducted on human patients suffering from visceral leishmaniasis due to infection with *Leishmania donovani* (*L. donovani*) showed that these patients had CD4^{+} T cells failing to express significant amounts of IFNγ and MIF, thereby failing to control infection. However, upon anti-leishmanial treatment and patient immunological recovery, MIF production was found to be restored (112).

One of the most important parasitic infection, if not the most important worldwide, is malaria, which is due to protozoan parasites from the genus *Plasmodium*. In 2019, there were an estimated 229 million cases of malaria and an estimated number of 409 000 deaths worldwide (113). Five *Plasmodium* species can cause disease in humans (*Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi*), but *P. falciparum* is by far the most prevalent causative agent of malaria in humans (113). The most common, often fatal, complications of *P. falciparum* infection are severe malarial anaemia (SMA) and cerebral malaria (CM) (64). The role of MIF in malaria infection and immunopathologies has been subjected to several studies. Experimental infections of BALB/c mice with *Plasmodium chabaudi chabaudi* (*P. chabaudi chabaudi*), which is a model for SMA, showed that an elevated plasma MIF concentration is associated with severe anaemia and impairment of erythropoiesis (114). Furthermore, *Mif* $^{-/-}$ mice infected with *P. chabaudi chabaudi* show less severe anaemia and a longer survival time, without any effect on parasitaemia, which is similar to the observations recorded in trypanosome infections (114). In human malaria infections, the exact contribution of MIF remains controversial. Several studies conducted on children infected with *P. falciparum* showed lower MIF concentration in infected children with severe complications compared to asymptomatic ones. These studies have suggested a protective role of MIF in malaria in general and SMA in particular (115, 116). However, other studies are in contradiction with this hypothesis, and rather suggest a link between higher MIF levels and severe malaria (114, 117, 118). Furthermore, additional studies suggest the implication of MIF in the pathogenesis of cerebral malaria, as higher MIF plasma concentrations were found to be associated with higher mortality in patients (119, 120).

Next to parasitic infections, MIF also plays a role in the modulation of the mammalian host microenvironment during other infections, such as bacterial infection. Indeed, MIF is known to play a crucial role in innate immune responses induced by lipopolysaccharide (LPS) and Gram-negative bacteria, by inducing the expression of Toll-like receptor 4 (TLR4) which is a component of the LPS receptor complex (66, 121). MIF has for example been shown to play a pivotal role in immunity against *Salmonella typhimurium*, as *Mif* $^{-/-}$ mice failed to control infection due to an impaired T_H1 response (122). Another study demonstrated that MIF-deficiency strongly impaired the killing of Gram-negative bacteria, such as *Escherichia coli* or *Klebsiella pneumoniae*, by macrophages. This was linked to a defective TLR4 signaling pathway, resulting in impaired activation of innate immunity (123). Also, the Gram-positive bacteria *streptococcus pneumoniae* infection strongly up-regulated MIF production and treatment with the anti-MIF antibodies significantly reduced bacterial loads and improved overall survival (124). For the intracellular *listeria monocytogenes* bacteria, the contribution of MIF depends on the pathogen dose, whereby the MIF titers increased 6h after lethal *L. monocytogenes* infection but not in the sublethal infection. The elimination of bacteria from the spleen and liver was not affected by anti-MIF antibody (Ab) injection in the sublethal infection, whereas the same treatment does rescued mice from the lethal infection indicating that in case of lethal *L. monocytogenes*, MIF can act as a susceptibility factor (125). Similarly, MIF is believed to be a critical mediator of the pathogenesis of endotoxic and septic shock, which are high incidence complications leading to mortality in emergency and intensive care units (ICUs) (66, 81, 82, 126, 127). In response to LPS, other bacterial components and/or stress, large quantities of preformed MIF are released and trigger the production of a strong pro-inflammatory environment that can culminate into septic/endotoxic shock due to uncontrolled TNF introduction. Furthermore, it has already been demonstrated that administration of MIF after LPS stimulation drastically increased mortality of mice (82). It has also been shown that serum MIF concentrations are higher in human patients with severe sepsis/endotoxic shock, and that these high levels correlate with disease outcome (128). Recently, a meta-analysis showed that blood MIF levels could have diagnostic ability to differentiate between infectious and noninfectious systemic inflammation and could have a bad prognostic value for the outcome of sepsis (129). Accordingly, approaches that could inhibit the action of MIF could have therapeutic potential. Indeed, a study reported that a panel of fully humanized anti-MIF antibodies directed against the binding epitopes within amino acids 50-68 or 86-102 of the MIF molecule, were protective in experimental models of sepsis (130). Also, small, affinity-matured nanobodies targeting MIF and engineered in a multivalent construct, were shown to attenuate lethality in a murine endotoxemia model after a lethal single injection of LPS (131). In addition, several inhibitors of MIF with potential therapeutic properties have already been developed and characterized (132).

Besides parasitic and bacterial models, in which MIF was shown to drive/mediate infection-related immune-pathological outcomes, analogous MIF-mediated effects were also documented to occur during experimental viral infections. For example, influenza A virus (IAV) infections in *Mif*$^{-/-}$ mice resulted in less inflammation, viral load and mortality compared to WT control mice and similar features were recorded with antibody-mediated MIF blockade (133). Conversely, transgenic mice overexpressing MIF in alveolar epithelial cells had higher inflammation, viral load, and mortality (133). Finally, treatment of human lung epithelial cells with recombinant human MIF promoted the spread of IAV *in vitro* (133). Collectively these studies reveal that during experimental influenza infection elevated MIF levels contributed to both inflammation as well as viral load. Consequently,

targeting MIF could be therapeutically beneficial in the treatment of influenza infections. In fact, treatment with the MIF small-molecule antagonist ISO-1 was documented to have a significant anti-inflammatory effect on avian H9N2 influenza virus-infected human lung alveolar epithelial (A549) cell inflammation and influenza M gene, coinciding with reduced viral load (134). Also treatment of H5N1 influenza virus infected mice with ISO-1 reduced significantly pulmonary cytokine production (135). Another example is the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for the COVID-19 disease. One of the hallmarks of this disease is the COVID-19 related macrophage activation syndrome (MAS), a condition characterized by overactivation of the immune system, leading to excessive pro-inflammatory cytokine production (136). This causes hyperinflammation and multiple organ failure, including the lungs, leading to many people requiring ICU hospitalization (137, 138). Being an important inducer of inflammation, MIF may be involved in the pathogenesis of COVID-19. Indeed, it has been shown that MIF levels positively correlate with disease severity in COVID-19, reflecting the inability of patients to control the increased pro-inflammatory cytokine production by anti-inflammatory mechanisms (138–140). These observations put MIF forward as a potential therapeutic target to treat patients with COVID-19 pneumonia.

Summary

MIF is a pleiotropic inflammatory cytokine controlling several processes essential for both innate and adaptive immunity. In general, there is an inverse correlation between MIF and pathogen control, however with exceptions such as African trypanosomes parasites, viruses and some bacteria, *e.g. Pseudomonas* and *Listeria monocytogenes*. However, if not controlled, this ubiquitously produced cytokine is also a potent inducer of systemic inflammation during different infectious and inflammatory diseases. As documented above, MIF plays an important role in the development of immunopathologies during experimental African trypanosome infection, including anaemia and liver injury. Furthermore, MIF is clearly linked to pathogenesis during other parasitic infections, such as Chagas' disease and malaria, or viral infections, such as COVID-19, and endotoxic/septic shock resulting from bacterial infection. Hence, the development of new therapeutic strategies directed against MIF could be an interesting approach to tackle these inflammation-associated immunopathologies. These strategies might be more effective in attenuating the induction/effects of a broad spectrum of pro-inflammatory cytokines rather that targeting each pro-inflammatory cytokine separately. Interestingly, many reports have documented a reciprocal interaction between MIF and GC hormones which will be briefly summarize in the next section.

THE MIF/GLUCOCORTICOID DYAD

Our immune system is able to regulate to some extent the pro-inflammatory immune response by producing glucocorticoid hormones (GCs, i.e. corticosterone (CT) in rodents and cortisol in humans) which are important to control sugar and fat usage by cells and curbing inflammation by eliminating chemicals involved in inflammation. Also during infections, the host enhances its production of GC hormones in an attempt to attenuate acute/excessive inflammation. For example, in case of African Trypanosomes HAT patients display higher serum levels of cortisol as compared to controls, probably reflecting a stress response to the ongoing inflammation (141, 142). Similarly, modulations of cortisol levels and GC synthesis were documented to occur during experimental Leishmaniasis and Chagas disease (143–145). More recently SARS-Cov-2 infection was suggested to affect GC synthesis since cortisol levels were found to be lower in critically ill patients with COVID-19 as compared to those of non-COVID-19 critically ill patients. Based on these findings the authors recommended measuring plasma cortisol to guide hormonal therapy such as systemic dexamethasone treatment (146). It should be emphasized that GC's represent the most important and frequently used anti-inflammatory drugs in routine clinical practice to treat a wide range of diseases, yet severe toxicity and irreversible side-effects often limits their use (147). Accordingly, the development of specific "Steroid sparing" therapies was considered to facilitate the action of GC's on inflammatory diseases by allowing dose reduction. In this context, MIF has emerged as a prime candidate to regulate GC sensitivity (148). Yet, treatment with GC is also associated with significant dose-dependent side effects. Indeed, unlike other pro-inflammatory cytokines, which are uniformly suppressed by glucocorticoids (GC), MIF expression can also be induced by GC (90). This induction is biphasic and concentration dependent, occurring maximally at low physiological concentrations such as 10−11−10−9M dexamethasone (77). Moreover, there is accumulating data suggesting that MIF functions as a counter-regulator of the anti-inflammatory effects of GC's since it sustains inflammatory responses in the fate of endogenous or exogenous GC's (90). Based on these findings the MIF/GC dyad was proposed as a physiological link that regulates immune and inflammatory responses. For instance, during parasitic infections such as African trypanosomes, a persistent induction of GC's may further fuel MIF production thereby undermining the beneficial anti-inflammatory activities of GC's and promoting GC resistance. The mechanisms underlying the anti-inflammatory action of GC's are multiple [reviewed in (149)], including the production of the immunosuppressive cytokines TGF-β and IL-10. Interestingly, depending on the cell types, MIF was reported to be able to regulate the expression of IL-10 and its receptor (150, 151). Together these findings may suggest that a triad MIF/GC/IL-10 can be operative during infections in the regulation of inflammation. Therefore, in the next section we will elaborate on the biological relevance of IL-10 as key regulator of the inflammatory cascade.

INTERLEUKIN-10 (IL-10)

Genetics, Receptor Signaling, and Functions of IL-10

The interleukin-10 (IL-10) protein is the founding member of the IL-10 family of cytokines, consisting of IL-10, IL-19, IL-20,

IL-22, IL-24, IL-26, and the less related IL-28A, IL-28B, and IL-29. This IL-10 family of cytokines has indispensable functions to maintain tissue homeostasis during infection and inflammation mainly through the restriction of excessive inflammatory responses, upregulation of innate immunity, and promotion of tissue repairing mechanisms (152, 153).

The IL-10 cytokine was identified for the first time in 1989 and described as a T_H2-secreted cytokine synthesis inhibitory factor (CSIF). This name referred to its ability to counteract the production of several pro-inflammatory cytokines produced by T_H1 cells, including IFNγ (154). Approximately one year later, the molecule was renamed IL-10 after both human and mouse CSIF cDNA had been cloned (155, 156). The human *IL-10* gene is situated on a conserved cytokine gene cluster on chromosome 1 (1q32) (157). Both murine and human IL-10 consist of 160 amino acids and are known to form functional noncovalently bound homodimers (152).

IL-10 can be produced by a large variety of innate and adaptive immune cells including macrophages, monocytes, DCs, granulocytes, NK cells, $CD4^+$ and $CD8^+$ T cells, and various B cell subsets (152, 158, 159). IL-10 production is regulated by different stimuli and regulatory mechanisms depending on the immune cell type (159). For example, innate immune cells such as macrophages and DCs, are activated through the recognition of pathogen-derived factors by pathogen recognition receptors (PRRs) on their cell surface, which triggers the expression of cytokines and other molecules (160). Examples of PRRs that have been associated with the induction of IL-10 expression in antigen presenting cells (APCs), are the Toll-like receptor 2 (TLR2) and TLR4 (161–163). After specific TLR ligation, signaling cascades are induced through different adaptor molecules, such as myeloid differentiation primary-response protein 88 (MyD88), ultimately leading to the production of IL-10 and other cytokines (162–164). Another important PRR in this context is the C-type lectin dectin-1, which regulates IL-10 production by macrophages for example, *via* a pathway dependent on the mitogen- and stress-activated protein kinase 1 and 2 (MSK1 and MSK2) and the cAMP response element-binding protein (CREB) transcription factor (165). In contrast, production of IL-10 by T cells is dependent on different transcription factors as well as cytokines depending on the T cell type. Examples include the Foxp3 and B lymphocyte-induced maturation protein-1 (Blimp-1) factors for both regulatory and effector type I regulatory (Tr1) T cells, respectively as well as the IL-27 cytokine for $CD4^+$ and $CD8^+$ effector T cells (166–170). Next to immune cells, IL-10 can also be produced by several non-immune cells such as hepatocytes and keratinocytes (48, 171, 172).

Once secreted, IL-10 protein carries out its functions through binding to the IL-10 receptor (IL-10R) on target cells. The IL-10R exists as a hetero-tetramer consisting of two IL-10R1 (IL-10Rα) subunits and two IL-10R2 (IL-10Rβ) subunits, which are both members of the interferon receptor (IFNR) family (159, 173–175). On one hand, IL-10R1 functions as the ligand-binding subunit, essential for binding of IL-10 with high affinity as well as in signaling (159, 174, 176). It is expressed constitutively mainly on leukocytes, *e.g.* T cells, B cells, NK cells, mast cells, and DCs, but can also be found on several nonhematopoietic cells upon induction (159, 173, 174, 176–178). On the other hand, IL-10R2 is an accessory, low affinity subunit that serves as an accessory chain essential for the active IL-10 receptor complex and to initiate IL-10-induced signal transduction events and it is expressed ubiquitously (159, 165, 179). Upon binding to its cognate receptor, IL-10 activates the Jak-STAT signaling pathway. This happens through phosphorylation-mediated activation of the Jak family tyrosine kinases Jak1 and Tyk2, which are associated with IL-10R1 and IL-10R2, respectively (180, 181). This ultimately results in the tyrosine phosphorylation of transcription factors STAT1, STAT3 and in some cases STAT5 (181–183). Through this pathway, IL-10 acts as a general suppressive cytokine directly inhibiting the production of pro-inflammatory cytokines such as IFNγ, TNF and IL-12 by immune cells. Furthermore, by lowering the antigen-presenting capacity of APCs and rendering them more suppressive, IL-10 indirectly inhibits T_H1 and T_H2 responses (42, 159, 184). This effect is reinforced by a direct effect of IL-10 on $CD4^+$ T cells, inhibiting their proliferation and cytokine secretion (185, 186).

Together, IL-10 prevents tissue lesions caused by exaggerated inflammatory immune responses (152). It has been shown that STAT3, but not STAT1, plays a key role in the function of IL-10 in immunosuppression and that IL-10-induced suppression of pro-inflammatory cytokine production was completely abolished in myeloid cells lacking STAT3 (187, 188).

Role of IL-10 in African Trypanosome Infection

As mentioned earlier, infection with African trypanosomes is associated with the development of a strong early pro-inflammatory immune response that culminates into severe immunopathologies and death if not tightly controlled. The IL-10 cytokine, with its anti-inflammatory character, was already shown to play a crucial role in counterbalancing this strong pro-inflammatory environment, thereby limiting pathology, but, in contrast, does not seem to modulate African trypanosome parasite control (42, 45, 48, 189–192). For example, IL-10 can dampen the differentiation of monocytes to Tip-DCs (TNF/iNOS-producing DCs) that have been shown to contribute to tissue damage during the chronic stage of *T. b. brucei* infection, *i.e.* pleomorphic AnTat1.1E model, thus limiting their production of pro-inflammatory cytokines such as TNF (42, 193). Accordingly, it has been demonstrated that IL-10-deficient (*IL-10*$^{-/-}$) C57BL/6 mice die from an uncontrolled pro-inflammatory cytokine storm within the first 10 days of a *T. b. brucei* infection, but this did not result in an incapacity to control parasite growth (42, 45). Similarly, *T. congolense*-infected *IL-10*$^{-/-}$ mice or mice treated with an anti-IL10R blocking antibody were shown to develop a hyper-inflammation syndrome and die after the first parasitaemia peak, without modulating parasitaemia peak in C57BL/6 mice (51, 190). It was found that the peak of IL-10 in circulation followed the peak production of pro-inflammatory cytokines required for first peak of parasitaemia control (45).

Many efforts have been put in the investigation of the relevant cellular sources, molecular mechanisms, and kinetics of IL-10 production during African trypanosome infection. Indeed, it was recently shown that IL-10 derived from hematopoietic cells is crucial to counteract an exaggerated production of pro-inflammatory cytokines during acute *T. b. brucei* infection, and thus prevent early mortality. Indeed, reconstitution of irradiated WT mice with IL-10-deficient bone-marrow cells resulted in comparable susceptibility to *T. b. brucei* infection compared to full *IL-10*$^{-/-}$ mice (45). Furthermore, using IL-10 reporter (Vert-X) mice, it has been found that NK cells, CD8$^+$ and CD4$^+$ T cells, B cells and plasma B cells represent potential cellular sources of IL-10 during the acute phase (first 10 days post-infection) of *T. b. brucei* infection (45).

The anti-inflammatory IL-27 cytokine has already been suggested to play a role in the induction of IL-10 production by effector T cells (166, 167, 194). However, during *T. b. brucei* infection and based on molecular and pharmacological inhibition, IL-27 plays a key anti-inflammatory role but apparently without directly acting on IL-10 production (45, 46). In contrast, T cell conditional knockout mice for the Blimp-1 transcription factor encoded by the *Prdm1* gene that was previously linked to the regulation of IL-10 production by T cells, display a significant decrease in the *T. brucei*-induced IL-10 in T cells. This conditional deficiency was linked to an uncontrolled pro-inflammatory cytokine storm associated to the premature death of these T cell-conditional *Prdm1* deficient mice as compared to *Prdm1* proficient controls linked (45, 170).

During *T. congolense* infection, *i.e.* Tc13 model, which is associated with a stronger and more sustained IL-10 production (48, 50, 192), additional leukocyte types have been found to produce IL-10. Indeed, both naturally occurring CD4$^+$ Foxp3$^+$ Tregs as well as different myeloid cell subsets, including Ly6C$^-$ patrolling monocytes and alternatively activated macrophages, were shown to constitute sources of IL-10 during the early stages of infection (49–51, 192, 195). Similar as for the *T. brucei* infection and using IL-27R deficient (WSX-1) mice, the IL-27 cytokine has been identified as a crucial anti-inflammatory cytokine limiting immunopathologies and prolonging survival of *T. congolense* infected mice, but again without directly acting on IL-10 production (46). With respect to the myeloid cells as potential IL-10 producing cells, the Ly6C$^-$ monocyte subset was shown to exert a hepatoprotective function in *T. congolense* infected mice by secreting IL-10 and by inducing, through cell-contact, the differentiation of pathogenic Ly6C$^+$ monocytes into macrophages expressing genes coding for anti-inflammatory molecules (*i.e.* alternatively activated macrophages) (41–43). Conversely, during *T. brucei* infection there is persistence of pathogenic Ly6C$^+$ monocytes that differentiate into classically activated macrophages that promote pathology development.

Recently, using IL-10 reporter (Vert-X) mice and albumin-specific *IL-10*$^{-/-}$ mice, it was also shown that non-hematopoietic cells, such as hepatocytes, constitute an important source of IL-10 during the chronic stage of experimental *T. congolense* infection required for controlling the development of immunopathologies, including liver injury, kidney failure and severe chronic anaemia (48). These results suggest that during *T. congolense* infection, an important switch in IL-10 producing cells takes place, from hematopoietic cells at the early stage to non-hematopoietic cells, such as hepatocytes, at the later stage of infection.

Together, the current knowledge put forward IL-10 as a master regulator of inflammation during African trypanosome infection. Therefore, approaches capable of increasing IL-10 concentrations could potentially restore the balance between pro- and anti-inflammatory responses and thereby control the development of severe immunopathologies. However, the cellular source of IL-10 regulating various immunopathologies is partly different between the experimental *T. brucei* and *T. congolense* model. In this context, treatment of *T. brucei*-infected mice with low doses of a CD28-specific superagonistic monoclonal antibody promoting IL-10 production *via* the expansion of Tregs and differentiation of alternatively activated macrophages resulted in an attenuated pathology and prolonged survival (59). Also, adenoviral delivery of IL-10 *via* an hepatotropic AAV vector that expresses the *IL-10* gene under the control of a hepatocyte-specific promoter, was shown to attenuate immunopathology and prolong survival of *T. brucei*-infected mice (**Figure 1B**) (42, 59). In addition, treatment of *T. congolense*-infected hepatocyte-specific IL-10 deficient mice with blocking anti-MIF antibodies resulted in increased IL-10 induction combined with an attenuated pathology and prolonged survival (48).

Finally, the limited clinical data available with respect to the role of IL-10 in HAT patients revealed that at later stages of the disease, when parasites enter the blood brain barrier (BBB), the levels of IL-10 in the cerebral spine fluid (CSF) are increased and correlate with increased levels of white blood cells and parasites within the CSF (196, 197). This observation was also confirmed in experimentally *T. b. rhodesiense* infected vervet monkeys as well as murine models whereby IL-10 and also IL-6 were found to protect the CNS from inflammatory pathology when parasites first enter the brain (196–198). Hence, it is suggested that the increased IL-10 levels within the CSF can be used as a stage marker and are required to reduce the severity of the neurological insult (197, 199). However, more studies using high throughput technologies, will provide a more detailed view of the exact role of IL-10 and/or critical molecules or pathways underlying the phenotype observed in human African trypanosome infection.

Role of IL-10 in Other Infections

During parasitic infection with *T. cruzi*, IL-10 has already been identified as a key cytokine, mediating an intimate balance between effective immunity and immunopathology. IL-10 production has been associated with susceptibility to *T. cruzi* infection, due to its ability to inhibit the killing of intracellular *T. cruzi* parasites by macrophages as well as macrophage production of pro-inflammatory cytokines involved in *T. cruzi* resistance, such as IL-12 and TNF (200–202). Thus, more IL-10 results in less inflammatory cytokines and thus higher pathogen

load. Furthermore, it was found that genetically susceptible mouse strains produced more IL-10 during *T. cruzi* infection than resistant mice, again suggesting a link between IL-10 and disease outcome (200, 203). Also, IL-10 was shown to be essential to avoid fatal systemic inflammation during *T. cruzi* infection governed by $CD4^+$ T cells and IL-12 during *T. cruzi* infection (204). Thus, these results suggest that IL-10 is a potent inhibitor of the resistance to *T. cruzi* as far as pathogen control is concerned, which contrasts the African trypanosomiasis model. Studies in human Chagas' disease patients have shown that a pro-inflammatory T_H1 immune response is key in the acute phase of the disease to control parasitaemia. However, at a later stage, it was shown that IL-10 production was necessary to restrict inflammation and avoid complications (205, 206). Indeed, asymptomatic patients were associated with a more anti-inflammatory cytokine profile with high expression of IL-10, while patients with cardiac complications were associated with high IFNγ and TNF levels. Thus, IL-10 production seems to require tight regulation, to be able to control inflammation while not being too immunosuppressive towards the cellular immune response needed for parasite control (205, 206).

Infections with Leishmania parasites, another member of the *Trypanosomatidae* family, result in a similar dual role of IL-10, whereby a trade-off must be made between effective parasite killing and avoidance of an exaggerated pro-inflammatory response. During *L. major* infection, the causative agent of cutaneous leishmaniasis, IL-10 diminishes T cell produced IFNγ and as a result, promotes parasite persistence (207, 208). Furthermore, complete parasite eradication was only obtained in resistant C57BL/6 mice with impaired IL-10 signaling, either by using *IL-10*$^{-/-}$ mice or by treating WT mice with anti-IL-10R antibodies (208). However, these C57BL/6 mice lacking IL-10 signaling were found to develop larger cutaneous lesions compared to controls, despite lower parasite burden. This increase in immunopathology in the absence of IL-10 was dependent on another pro-inflammatory cytokine, namely IL-17, promoting neutrophil recruitment (209). Also, they showed that IL-10 and IFNγ regulate the IL-17 responses of peripheral blood mononuclear cells (PBMCs) from human patients with leishmaniasis In addition, although having better parasite clearance, the *IL-10*$^{-/-}$ mice displayed a loss of immunity to reinfection, suggesting a role of IL-10 in the maintenance of effector memory responses (207). During *L. donovani* infection, leading to visceral leishmaniasis, depletion of IL-10 was also shown to induce increased production of IL-12 and IFNγ. This reinforced pro-inflammatory response was accompanied with exaggerated granuloma formation and increased parasite elimination (210–212). In human patients suffering from visceral leishmaniasis, IL-10 is also suggested to operate as a double-edged sword. The elevated levels of IL-10 found in serum of patients with active disease as well as the enhanced IL-10 mRNA expression in lesional tissue, could help limit immunopathologies but could also promote parasite proliferation and disease progression (213).

In malaria infections, IL-10 has been found crucial to mediate the balance between pro- and anti-inflammatory responses. This balance is fundamental to eliminate the plasmodium parasites and at the same time to prevent severe malaria complications, including severe anaemia, cerebral malaria, and multiple organ failure (214, 215). Indeed, infection of *IL-10*$^{-/-}$ mice with *P. chabaudi chabaudi* led to exacerbated pathology which was linked to increased expression of pro-inflammatory cytokines such as IFNγ, IL-12 and TNF (216). This phenomenon was also observed in T cell specific *IL-10*$^{-/-}$ mice infected with *P. chabaudi chabaudi*, which behaved similarly as full *IL-10*$^{-/-}$ mice (217). Furthermore, IL-10 was suggested to have a protective role in experimental cerebral malaria, caused by *Plasmodium berghei*, as decreased IL-10 mRNA expression in spleen and brain correlated with increased susceptibility to infection (218). In contrast, IL-10 production was shown to be linked to a strong inhibition of pro-inflammatory responses resulting in high parasitaemia in *Plasmodium yoelii* infection in mice (219). It has also been linked to the development of hyper-parasitaemia in mice infected with *Plasmodium chabaudi adami* (220). Together, these results from mouse models of malaria suggest that IL-10 is needed to protect from excessive tissue inflammation, but thereby also promotes parasite proliferation. Studies in humans have suggested a role of IL-10 in regulating the pathogenic effects of TNF during malaria (215). For example, a study conducted in Western Kenyan children showed that higher ratios of plasma IL-10 to TNF levels were strongly linked to protection against severe malaria anaemia (221). Similar to studies in pre-clinical models, African children with severe anaemia had lower plasma IL-10 levels than patients with moderate anaemia or cerebral malaria, suggesting that IL-10 plays an important role in preventing severe anaemia (222). However, by conducting this protective role, IL-10 could also lead to high parasite-density infections with the development of other complications, such as accumulation of parasitized RBCs in tissues leading to hypoxia and vasculature damage (215). Furthermore, not all field studies support the hypothesis that IL-10 controls severe anaemia. Indeed, high levels of IL-10 have already been linked to increased disease severity in humans, including for example cerebral malaria and lung pathology called malaria-associated acute respiratory distress syndrome (MA-ARDS) (223, 224).

In the context of bacterial infections, the first suggestions on the role of IL-10 in controlling inflammation came after several observations in *IL-10*$^{-/-}$ mice, which developed spontaneous colitis in response to their modified gut flora (68, 225). For actual infections with bacterial pathogens, the contribution of IL-10 was shown to be dependent on the nature of the infective bacteria, and more particularly on two specific aspects: first, whether the bacteria live intracellular or extracellular in the host, and secondly, the magnitude of the pro-inflammatory immune responses elicited during infection (226). Accumulated data suggest that for extracellular and/or highly pro-inflammatory bacteria, IL-10 production is crucial for limiting tissue damage and improving host survival. Examples are the extracellular bacteria *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*, which all trigger an important pro-inflammatory immune response, whereby IL-10 is crucial to keep this in balance to avoid severe complications and organ

damage (225–231). The same applies for some intracellular bacteria, *e.g. Francisella tularensis*, known to trigger a strong pro-inflammatory response (232, 233). Also, collected data suggest that for another intracellular bacteria, namely *listeria monocytogenes*, IL-10 impedes effective bacterial clearance and promotes pathogen dissemination (234, 235). Similarly, IL-10 was shown to have a detrimental impact on the clearance of pulmonary *Klebsiella pneumoniae* and *Bordetella pertussis*, the causative agent of the whooping cough (236). These are typically extracellular bacteria, but which were shown to evade the immune system by hiding intracellularly and by suppressing the inflammatory immune response (237–240). Therefore, the IL-10 produced by the host during these infections could act in synergy with the intrinsic ability of these pathogens to undermine the immune response, thereby further promoting bacterial proliferation and dissemination. In this context, administration of exogenous IL-10 to mice and human patients showed potent inhibitory effects on the development of inflammation resulting from endotoxemia (241, 242). Also, in human patients with meningococcal septic shock, it was found that IL-10 was responsible for the main monocyte inhibiting characteristics in the circulation, which reinforces the hypothesis on the anti-inflammatory role of IL-10 during clinical bacterial infections (243).

During viral infections, the exact role of IL-10 is also often unclear and depends on different factors such as, the virus type, the site of infection and the timing of the immune response. IL-10 is known to be critical to protect the host from excessive tissue inflammation during acute viral infection (244). At the same time, it was linked to increased virus persistence at a later stage during HIV infection (245). IL-10 was also shown to have a detrimental effect on the humoral immunity during acute influenza A virus infection, since *IL-10*$^{-/-}$ mice had improved viral clearance compared to WT mice (246). However, IL-10 produced in large amounts by antiviral effector T cells was crucial to limit pulmonary inflammation and avoid lethal injury during acute influenza infections (247). A unique feature of the COVID-19 induced cytokine storm compared to other SARS-CoV viruses, is a drastic elevation in IL-10 in critically ill patients (248, 249). Indeed, IL-10 concentrations are elevated in patients in ICUs (250, 251). These elevated IL-10 levels were first suggested to be an anti-inflammatory or immune-inhibitory mechanism that serves to counteract the important release of pro-inflammatory cytokines (251, 252). However, recent clinical evidence put forward the hypothesis that this dramatic increase in IL-10 levels might play a role in the pathology of COVID-19 (252). It is suggested that the exacerbated IL-10 production might work as an immune activating agent by for example stimulating the production of other factors of the cytokine storm, thereby amplifying the viral sepsis-related hyperinflammation in critically ill COVID-19 patients (252, 253). This potential pro-inflammatory promoting character of IL-10 has already been observed during human endotoxemia (254).

Summary

IL-10 is a pleiotropic anti-inflammatory cytokine crucial to control pro-inflammatory responses during infections, thus preventing inflammatory pathologies. For example, IL-10 is put forward as a master regulator of inflammation during African trypanosome infection, being key to limit the development of severe immunopathologies. However, IL-10 levels do not seem to affect African trypanosome control. Evidence from other parasitic infections have confirmed this role of IL-10 in limiting inflammation and pathology but have also put forward that this is often accompanied with decreased pathogen clearance (in contrast to trypanosomiasis). Indeed, IL-10 is often implicated in a see-saw balance between effective immunity to clear the pathogens and the avoidance of immunopathology development. For bacterial and viral infections, the exact contribution of IL-10 is strongly dependent on different aspects such as pathogen type and infection site.

CONCLUSION

The present literature survey documents extensively the opposite activities of the polarising cytokines MIF and IL-10 during infections with various extracellular and intracellular pathogens, but with a special emphasis on parasitic disease. While in general infection-associated MIF promotes a pro-inflammatory response that may contribute to pathogen control, sustained inflammation can cause collateral tissue/organ damage. Such MIF-mediated pathological side-effects during infection can be alleviated by pathogen-elicited IL-10 production. Accordingly, during infections a balanced equilibrium between MIF (pathogen control) and IL-10 (pathology control) may confer tolerance towards an infectious disease (**Figure 2**). In contrast, in most infectious settings, an unbalanced MIF/IL-10 ratio may lead to either higher pathogen loads/lower pathology (MIF^{low}, IL-10^{high}) or lower pathogen loads/excessive pathology (MIF^{high}, IL-10^{low}).

This molecular "Yin-Yang" between the dyad MIF/IL-10 was extensively investigated in experimental Trypanosomosis using susceptible (*T. brucei*) and tolerant (*T. congolense*) models. Based on these investigations (summarized in **Figure 1**) susceptibility versus tolerance towards African trypanosomes reflects a MIF/IL-10 balance namely MIF^{high}/IL-10^{low} (susceptible phenotype) versus MIF^{low}/IL-10^{high} (tolerant phenotype). Selective interference with this balance allows to switch between these two phenotypes as illustrated in **Figure 1**. Of note, the model proposed in **Figure 1** is based on serum levels, and does not consider certain tissue microenvironments, such as the liver and adipose tissue, which may be relevant in the control/chronicity of infection with *T. brucei*. Furthermore, the model proposed for *T. brucei brucei* and Tc13 might not necessarily reflect results from other types of *T. brucei* subspecies (e.g., *T. gambiense*) and *T. congolense*, respectively. Extrapolation of this physiological MIF/IL-10 model to other pathogens/infections deserves also some caution. Indeed, though MIF drives inflammatory responses during African trypanosome infections that are supposed to control parasite development, absence (MIF KO mice) or blocking (anti-MIF Abs) of this cytokine did not affect the parasitaemia levels. Several factors acting either alone or in

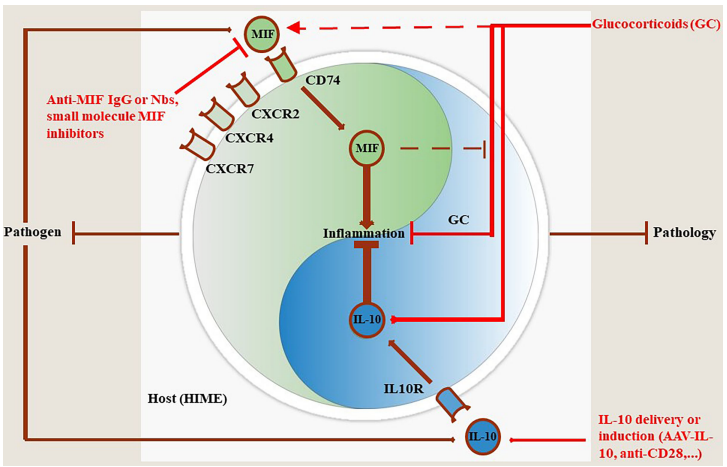

FIGURE 2 | MIF and IL-10 act as a molecular Yin-Yang rheostat in the modulation of the Host Immune Microenvironment (HIME) during pathogen infection. Most pathogens trigger MIF production during the early stages of infection that drives pro-inflammatory immune responses involved in the control of most pathogens, except African trypanosomes. Binding of MIF to its receptor CD74 triggers pro-inflammatory immune responses, while binding to CXCR2, CXCR4 and CXCR7 initiates immune cell recruitment to fuel and sustain this immune response (see MIF section). To compensate and prevent excessive tissue injury during infection, the host can mount an anti-inflammatory immune response in which IL-10 plays a key role in pathology control. If this balance is maintained throughout infection animals will exhibit a phenotype characterized by efficient pathogen control and pathology control. Conversely, if the balance is tilted in favor of MIF infected animals will exhibit a phenotype characterized by efficient pathogen control coinciding with tissue injury and early death resulting in pathology-mediated host death. When the balance is skewed towards IL-10 the animals will exhibit a phenotype characterized by limited pathogen control coinciding with limited pathology resulting in host death, but due to uncontrolled pathogen load in this case. Treatment with glucocorticoids (GC) can dampen inflammation through various mechanisms including induction of IL-10 transcription. However, GC can also induce MIF which in turn overrides the anti-inflammatory activity of GC. In such situations the efficacy of GC treatment is hampered and, therefore, MIF blocking strategies or IL-10 delivery (AAV)/induction (anti-CD28) may be appropriate.

concert may account for this discrepancy. First, the remaining/reduced levels of inflammatory cytokines present under these conditions are sufficient to control parasitaemia. In this context, it is currently unknown what are the minimal levels of pro-inflammatory cytokines required to control parasitaemia development. Secondly, the antibody response raised upon infection could be sufficient for parasitaemia control (33, 255). Thirdly, the expression of CRIg, i.e. a complement receptor essential in capturing bloodborne pathogens through complement opsonization and shown to play an important role in intravascular clearance of African trypanosomes (256), which is typically suppressed upon inflammation, might be less affected and therefore allow sufficient parasite elimination. Fourthly, the density-dependent quorum sensing (QS) mechanism is sufficient for parasitaemia control at least for the experimental *T. brucei brucei* model (257).

When evaluating the role of MIF or IL-10 on the course of other infections it is clear from many reports that interfering with either MIF or IL-10 affects differentially the pathogen burden. Indeed, while in general MIF inhibition results in higher pathogen load the opposite occurs when IL-10 is blocked, namely lower pathogen loads. Such effects could be pathogen dependent as illustrated for African trypanosomes and should be taken into consideration when applying therapeutic interventions based on MIF antagonism.

The mechanisms underlying the interactions between MIF and IL-10 *in vivo* remain to be determined. For example, it was shown *in vitro* that IL-10 can inhibit the induction of MIF by T-cells and abolish MIF-mediated monocyte migration through so far unknown mechanisms (151). In contrast, depending on the cell type, MIF has been shown to regulate *in vitro* the synthesis and expression of intracellular IL-10 by peritoneal cells as well as an increase of the IL-10 receptors on the surface of Kupffer cells (150). Hence, at least *in vitro* IL-10 and MIF regulate each other. A possible link might rely on glucocorticoids since these have been found to induce IL-10, yet at lower concentrations they were also found to be able to trigger MIF production (**Figure 2**). In turn, MIF can override the effects of glucocorticoids thereby further promoting a pro-inflammatory immune response. In this context, it was observed that MIF suppresses IL-10 production and down-regulates the serum cortisol level during a lethal infection with *L. monocytogenes* (125). However, so far, no thorough studies that directly examine MIF effects on IL-10 expression under the influence of GCs have been performed and this warrants further research. Altogether, future intervention strategies aiming at restoring the timely and delicate balance between pro- and anti-inflammatory signals could be key to alleviate immunopathology development and promote survival. In this regard, strategies that specifically block MIF signaling (through for instance monoclonal antibody treatment or small molecule MIF antagonists) and/or promote IL-10 signaling pathways, for example *via* IL-10-AAV or anti-CD28, might be considered as potential therapeutic candidates not only during African trypanosome infections (see **Figure 1B**), but also for other diseases and under non-infectious settings. However, therapeutic intervention strategies for MIF and IL-10 might

not exert a similar effect in all disease settings. For instance, *in vitro* blockade of MIF was also found to inhibit in an autocrine manner IL-10 production, on top of TNF, by RSV-infected macrophages (258). Hence, in these settings MIF blockade might have adverse effects. Also, IL-10 is typically seen as an anti-inflammatory cytokine required to dampen pathology. Yet, during lethal *L. monocytogenes* infection, IL-10 was found to exert anti-bacterial activity by promoting macrophage activation (125). Also during COVID-19 infection, increased levels of both MIF and IL-10 were found to independently correlate with severity and pathology. Finally, high levels of MIF and IL-10 were both found to have bad prognostic value for the fatal outcome of sepsis patients, where high levels of IL-10 were found to have the highest prognostic value (254, 259, 260). In each of these cases, IL-10 was shown to exert also pro-inflammatory rather than solely anti-inflammatory effects and thereby further contribute to aggravation of the disease. Moreover, treatment of T_H1 mediated illnesses using high doses of IL-10 might have adverse effects and rather trigger IFNγ-mediated detrimental effects (254).

Collectively, the MIF and IL-10 dyad could be considered as a potential novel molecular Yin-Yang in infectious disease progression. However, this molecular rheostat will mainly depend on i) the type and level of infection, ii) the cytokine environment at that stage of infection and iii) the affected cell type. Together, an appropriate and "personalized" strategy directed against MIF, IL-10 or both should be considered to alleviate pathology without compromising pathogen control within a particular pathogen context (**Figure 2**).

AUTHOR CONTRIBUTIONS

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

FUNDING

The authors acknowledge the financial support of the Interuniversity Attraction Pole Program (PAI-IAP N. P7/41, http://www.belspo.be/belspo/iap/index_en.stm) as well as grants from the FWO (FWO G015016N), the Strategic Research Program (SRP#3, SRP#47 and SRP#63, VUB), the National Institute of Health (NIH) (R21CA259345) and the VUB OZR mandate (OZR3833). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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frontiers | Frontiers in Immunology

REVIEW
published: 29 April 2022
doi: 10.3389/fimmu.2022.869163

The Influence of Genetic and Environmental Factors and Their Interactions on Immune Response to Helminth Infections

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Helminth infection currently affect over 2 billion people worldwide, with those with the most pathologies and morbidities, living in regions with unequal and disproportionate access to effective healthcare solutions. Host genetics and environmental factors play critical roles in modulating and regulating immune responses following exposure to various pathogens and insults. However, the interplay of environment and genetic factors in influencing who gets infected and the establishment, persistence, and clearance of helminth parasites remains unclear. Inbred strains of mice have long been used to investigate the role of host genetic factors on pathogenesis and resistance to helminth infection in a laboratory setting. This review will discuss the use of ecological and environmental mouse models to study helminth infections and how this could be used in combination with host genetic variation to explore the relative contribution of these factors in influencing immune response to helminth infections. Improved understanding of interactions between genetics and the environment to helminth immune responses would be important for efforts to identify and develop new prophylactic and therapeutic options for the management of helminth infections and their pathogenesis.

Keywords: genetics, environment, interaction, Helminth infection, heterogeneity

OPEN ACCESS

Edited by:
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National Institutes of Health (NIH), United States

Reviewed by:
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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *03 February 2022*
Accepted: *04 April 2022*
Published: *29 April 2022*

Citation:
Oyesola OO, Souza COS and Loke P (2022) The Influence of Genetic and Environmental Factors and Their Interactions on Immune Response to Helminth Infections. Front. Immunol. 13:869163. doi: 10.3389/fimmu.2022.869163

INTRODUCTION

Research into factors that influence host response during helminth infection are usually focused on use of inbred mice raised in a specific pathogen free environment as found in most research institutes and academic institutions across the world. However, in the real-world setting, helminth infections are characterized by infections of individuals living in various communities with different lifestyles as well as with wide genetic variations. Also, the intensity of helminth infection among individuals varies markedly and can be influenced by various genetic and environmental factors (1, 2). Therefore, in this review, we discuss the influence of genetic and environmental diversities in the regulation of helminth induced immune response and their contribution to inter-individual variation seen in responses during helminth infection (3, 4). Furthermore, we highlight ongoing studies and future opportunities to examine the interaction between environment, genetics and other variables that influences the interindividual variation seen during helminth infection.

IMMUNE RESPONSE TO HELMINTH INFECTION

Studies with genetically modified mice on the C57BL/6 background has transformed our understanding of Type 2 responses to helminths in the last few decades. Immune responses during helminth infection are characterized by recruitment and accumulation of innate immune cells such as eosinophils, basophils, innate lymphoid cells (ILC2), neutrophils, alternatively activated macrophages as well as cell of the adaptive immune system such as B cells, Th2 and T regulatory CD4 T cells (5–11). These cells produce Type 2 and regulatory cytokines and other mediators which play important protective and regulatory functions during helminth induced inflammation (12–14). Recent advances demonstrate the critical role other previously overlooked cells such as epithelial, neuronal, and stromal cells play in contributing to and regulating Type 2 immune responses during helminth infection (5, 15, 16). These non-immune cells can produce cytokines, alarmins and other bioactive mediators that crosstalk with innate and adaptive immune cells to regulate the response during helminth infection. Despite these advances, inter-individual variation in these responses and the influence of the environmental interaction with host genetics in free-living mammals is not well understood.

GENETIC VARIATION IN RESISTANCE TO HELMINTH INFECTION

Our ability to genetically manipulate mice has been fundamental for increased understanding of mammalian physiology. As this technology evolved over time, the C57BL/6 strain of mice has emerged as the inbred strain of choice for most immunological studies. As a result, our understanding of the basic mechanisms that surround immune response during helminth infection has also improved significantly with the use of genetically modified mouse models for dissecting various mediators and immune cell populations in the regulation of helminth induced immune responses. Increasing complexity of mouse models include cell specific knockouts, genetic inducible fate mapping models, in addition to the global knock-out and/or transgenic mice models have been critical in identifying new pathways that regulate Type 2 and immunoregulatory responses to helminths. To reduce variation in these reductionist experiments to characterize detailed mechanisms, genetically identical mice strains of similar age groups and sometime sex are used to isolate and study the role of specific cell types and immune mediators.

However, various other studies have used in-bred strains of mice to study the role of genetic variations in resistance to helminth infection (17). For example, previous studies have shown that the BALB/c strain of mice are more susceptible to *Litomosoides sigmodontis*, a filarial nematode, compared to the C57BL/6 mice or the C57BL/10 mice (18–20). This contrasts with other intestinal helminth parasites such as *Trichuris muris* (21–23) and *Heligosomoides polygrus* (24–26) where the BALB/c strain has been shown to be more resistant. Other studies have also examined inbred strains with various other helminth parasites (**Table 1**). While genetic variation clearly influences susceptibility and resistance to helminth infection in mouse models, our understanding of the basic mechanisms elicited during helminth immune response critical for mediating these differences remain unclear. While mechanisms driving these differences include the role of various effector immune cells, cytokines, and immunoglobulins (**Table 1**), the role of genetic variation in regulating primary and/or secondary sentinels of Type 2 inflammation (55–57), such as epithelial, stromal, and neuronal cells is currently less appreciated and has not been well studied. For example, in the area of epithelial cell biology, C57BL/6 and BALB/c mice show differences in tuft cell response at steady state and in response to a protozoa parasite, *Trichomonas muris*, but no significant difference was seen in tuft cell response following chronic infection with *H. polygyrus* at peak of parasite establishment (58). The dynamics of tuft cell hyperplasia in the different inbred strain of mice could vary wherein the BALB/c mice might have higher response than the C57BL/6 mice (59). Hence, the role of these sentinels in the pathogenesis and outcome to helminth infection should be examined in different inbred strains of mice.

Studies in other free-living mammals such as the livestock population and wild animals has also highlighted the importance of genetic factors in susceptibility to helminth infection (60–64). For example, farmers and livestock breeders will often use their knowledge of breed specific resistance and susceptibility to helminth infection to minimize cost and losses associated with infection with helminths, by selecting helminth parasite resistant strains for breeding. Some studies have linked these genetic resistant and susceptibility patterns to the protective Type 2 mechanism (60, 64, 65).

There are fewer studies in the human population that provides a mechanistic understanding to the influence of genetic variations on susceptibility to helminth infection, despite substantial evidence for the role of genetics in determining susceptibility to infections (1). Logistical and ethical constraints often limit human population studies to correlational observations rather than a study of cause-and-effect relationships. Currently, host genetics is said to account for about 20 to 40% of variation in intensity of worm burdens seen during helminth infections (1). For example, a few studies have demonstrated the role of genetic factors in susceptibility to human Ascaris infection (66–68). Notably, they were able to associate this genetic factor to a peak in chromosome 13 which is close to the known locus of a major candidate gene, *TNFSF13B*, involved in the regulation of B cell activation and immunoglobulin secretion (68–71). A few other genetic factors such as during *Trichuris trichiura* infections have also been identified (72) and associated with localization of two significant quantitative trait loci on chromosomes 9 and 18, which contains genes that can influence immunoregulatory cytokines like IL-10 (73). Susceptibility to other helminth parasites such as blood flukes like *Schistosoma mansoni* and hookworms like *Necator americanus* and *Ancylostoma duodenale* has also been linked to genetic factors (74–78). What is unclear is when linkage of susceptibility to helminth infection involves more than one genetic locus, whether this is dependent

TABLE 1 | Understanding the role of naturally occurring genetic variation in resistance and outcomes to helminth infections – mice models.

Genetic Susceptible strains	Helminth infection	Type of Helminth Parasite	Protective/Susceptible mechanistic explanation	References
BALB/c	*Litomosoides sigmodontis*	Filarial Parasites	CD4 T lymphocytes; production of IL-4	(18–20, 27)
AKR; B10.BR	*Trichuris muris*	Whipworm	Higher Th1 effector response characterized by increased IFN gamma production	(21–23, 28–31)
CBA; C3H; SLA/J; C57BL/6; C57BL/10	*Heligmosomoides polygyrus*	Hookworm	Decreased Th2 driven effector response characterized by lower IgE responses, lower intestinal mast cell densities, alternatively activated macrophages and a concomitant increase in TNFα and IFN γ response; Increased proportion of CD103+FoxP3+ activated T Regulatory cells in susceptible strains;	(24–26, 32–37)
C57BL/6	*Ascaris suum*	Round worms	Hepatic factor, less intense inflammatory and repair response in the liver? Role of secretory IgA	(38–40)
CBA; BALB/c; C57BL/6;	*Nippostrongylus brasiliensis*	Round worms	Developmental arrest in the lungs or migration deficiency of larva into the intestinal tissue in resistant mice, FVB/N; Immunological mechanism is not clear, possibly a Type 2 dependent immune response that limits tissue associated immune response	(41–43)
BALB/c, DBA/2	*Taenia crassiceps*	Tape worms	T cell dependent mechanism. Role of Regulatory T cells	(44–48)
C57BL/6	*Trichinella spiralis*	Round worms	Mucosal mast cells	(49)
CBA; C57BL/10	*Schistosoma mansoni*	Blood flukes (trematodes)	Increased IL-1β and IL-23 cytokines by DCs and T helper 17 polarization; Proinflammatory T helper 1/ T helper 17 responses persist along with T helper 2; Reduction of the alternative activation marker	(50–54)

on one gene or if genetic variation in other genes could affects resistance to helminth parasite. Perhaps mechanistic insights could be gleaned from mouse models, if these regions are conserved, to understand the relationship and function of those genes. Therefore, such clinical and genetic epidemiology studies may guide the conduct of fundamental and basic immunology experiments.

Host genetics could also indirectly influence other host associated factors which then indirectly influence the immune system. For example, difference in expression of major histocompatibility complex molecules can influence the composition of the gut microbiome (79–82) through differences in antibody responses against commensal bacteria (80). Incidentally, these MHC associated differences in gut microbiome may then influence subsequent susceptibility to helminth infection (79). Hence, genetic factors may alter the host microenvironment to affect subsequent outcomes to helminth infections. However, because these are observational studies in primates, it is challenging to mechanistically isolate the effect of immune mechanisms resulting from different MHC haplotype and effects due to microbiome differences.

GENETIC VARIATION IN PATHOGENESIS OF HELMINTH INFECTION

It is important to distinguish between the role of genetic variation in resistance and pathogenesis to helminth infections. Although resistance and pathogenesis are linked because disease morbidity is often observed in heavily infected individuals, mechanisms that drive disease pathogenesis may be unrelated to mechanisms responsible for parasite resistance. Our understanding of pathogenesis during helminth infections is based primarily on studies with genetically modified mouse models, which provide insights into the pathways, cytokines and other mediators that regulate the disease processes. Also, differences in disease outcomes from infection of inbred strains of mice have provided additional clues in the heterogeneity of immune response and severe pathology.

The cytokine balance during infection is an important determinant of pathogenesis, mediating both resistance and tolerance to infection. Rapidly after infection, pathogen associated molecular patterns are detected by pattern recognition receptors (PRRs) together with release of alarmins like IL-33, IL-25 and TSLP at epithelial barriers (83, 84). This leads to activation of transcription factors, such as STAT6 and GATA3, which subsequently induce the upregulation of sets of genes including receptors, cytokines, chemokines, and genes regulating the production of eicosanoids (85–87). Cytokines, chemokines, and eicosanoids can induce recruitment, accumulation, and differentiation of immune cells with release of additional sets of effectors cytokines that then induce repair, differentiation, and release of effector molecules from the epithelial barrier to help in the clearance of the worms (87–90). Thus, cytokines, chemokines, eicosanoids, and other mediators are key in the initiation as well as in the pathogenesis of anti-helminth immune responses.

Alterations to the balance of the cytokine response because of host genetic variation can affect the inflammatory mediator profile which can determine the pathogenesis of helminth infection. For example, C57BL/6 and CBA mice show different levels of immunopathology during *S. mansoni* infection, when challenged with a similar number of cercariae. This may be due to differences in the switch from a pro-inflammatory T helper 1 (Th1)/T helper 17 (Th17) response to a tissue protective Type 2 response. The CBA mice show higher levels of proinflammatory Th1/Th17 cytokines which does not diminish but instead persists alongside the rising T helper 2 (Th2) responses, while C57BL/6 mice can regulate the Th17 response during the expansion of Th2 cells resulting in milder

pathology (50–52). However, it is notable that a prolonged Th2 response may lead to other forms of pathology such as increased fibrosis in the BALB/c mice (91). Similarly, *S. mansoni* infection in humans can have different outcomes and pathophysiology, ranging from the mild symptoms to severe symptoms such as the development of severe hepatic fibrosis, hepatosplenic disease, ascites, and encephalopathy, irrespective of the intensity of infection (77, 78). In some cases, a genetic explanation has being proposed in individuals with severe pathological outcomes with some evidence of polymorphisms in cytokine and cytokine receptor genes like *IFN-γR1* gene, which encodes the receptor for the Type 1 cytokine, IFN-γ (78, 92), those involving *TGFBR2* which encodes for receptor of regulatory cytokine, TGF-β (78) and *IL-22RA2*, which encode the receptor for IL-22 (93). Other genes such as polymorphisms in *CNN2* gene, which encodes for the connective tissue growth factor (CTGF), a stromal factor, has also been implicated in the pathophysiology of these disease (94, 95).

Resistance to helminth infection in host of different genetic backgrounds may also be associated with roles of different cytokines in regulating the outcome to infection. For example, differences in susceptibility to *T. muris* infection in C57BL/6 mice compared to BALB/c mice might be due to the role of different cytokines with IL-4 playing a predominant role in C57BL/6 mice and IL-13 playing a more predominant role in BALB/c mice (96). In addition, the protective immunity in C57BL/6 mice vaccinated with radiation-attenuated (RA) larvae of *S. mansoni* is associated with Th1 immune response, while on BALB/c background the protection depends on Th2 responses. Here, injection of RA-attenuated larvae produced lower levels of IgG1 antibodies in serum IL-4Rα deficient mice (IL-4Rα$^{-/-}$) on BALB/c background, but the serum from vaccinated wild-type BALB/c mice confers protection to IL-4Rα$^{-/-}$ mice, suggesting the Th2 antibodies is crucial for parasite elimination and resistance in BALB/c mice (97).Genetic differences due to a distinct pattern of cytokines secreted and markers expressed by myeloid cells can also correlate with different helminth infection outcomes. For example, dendritic cells (DCs) from *S. mansoni*-infected high morbidity CBA mice display increased expression of CD209a (C-type lectin receptor – CLRs) which is necessary for the production of IL-1β and IL-23 that drives pathogenic Th17 cells, as opposed to the low morbidity C57BL/6 mice (51, 52, 98). Similarly, C57BL/10 mice develop more severe schistosomiasis, as defined by significant larger granulomas, increased proinflammatory cytokines production by DCs and higher levels of IL-17 compared to C57BL/6 mice. This phenotype is tightly connected to DCs function, because DCs from C57BL/6 mice expressed high levels of Ym1 and RELMα, marker of alternative activation that regulates the tissue repair in responses to *S. mansoni* eggs (53).

Population genetics studies in humans have shown that the number of pathogens in a specific geographic region can have selective pressure on genes related to cytokine production and responsiveness (99–101), regulation of cellular responses (102) as well as transcription factors important for the induction of protective Type 2 immune response during helminth infection (103, 104). This suggest that evolutionary pressure in different geographic location with different parasite profiles can influence polymorphism of genes and regulatory elements that can then affects response to parasites endemic in those regions.

Parasite Factors as Source of Variation to Host Responses During Helminth Infection

In addition to host genetic factors, variation in parasite species and genetics can also be a major contributor to heterogeneity in susceptibility and resistance patterns during helminth infection (105–108). For instance, variation in reproductive output and consequently egg production following helminth infection has been attributed to parasite genetic factors (108). Parasite genetics could also influence the properties of excretory and secretory proteins released from the worms, which could in turn influence host-parasite interactions as well as parasite chronicity patterns (109, 110), the ability of the parasite to evade the host immune system (110) or the pathogenesis of immune response in the host (107, 108, 111–114). For example, differences in lifecycles and egg properties between *S. haematobium* and *S. japonicum* results in varied cellular and humoral immune responses despite belonging to the same helminth parasite genus (108, 111, 112).

Moreover, different stages of the lifecycle are also an important source of heterogeneity in host responses. As seen in *S. mansoni*, there is a clear shift from a Th1 mediated immune response to a Th2 immune response at the onset of egg production (115), while the cercariae and larva induce Th1 dominated immune response (116). The different lifecycle stages also produce different suites of excretory/secretory products that modulate the host response in diverse ways to promote invasion, infection, adhesion and the immunoregulatory process (108, 117, 118). Hence, parasite factors are a critical source of heterogeneity in immune responses during helminth infection.

Furthermore, in mouse models of infection, strain specific differences have also been described. Strains of *T. muris* isolated and maintained in different parts of the world, including the S strain (isolated in Sobreda, Portugal), the E strain (isolated in Edinburgh) and the E-J strain (originally E strain, which has been maintained in Japan since 1969) can induce different immune response following infection of mice. The E and E-J strains of *T. muris* generally induce a Th2 skewed response whereas the S-strain induces a Th1 skewed response characterized by production of IFN-gamma and IL-12 (107, 113, 114). Therefore, heterogeneity in parasite genetics can be a source of variation in immune response and perhaps helminth parasites can adapt their metabolism and alter the immune response to fit into their environment (109, 119). Since most laboratory strains have been maintained and passaged in different laboratories for several years, it is possible that parasites used in most laboratories diverge significantly from wild helminth parasites that are present in their natural environment.

ROLE OF ENVIRONMENT DURING HELMINTH INDUCED TYPE 2 IMMUNE RESPONSE

There are many environmental variables that can affect parasite burden in an endemic population and dissecting the relative

contribution of each variable can be difficult. Most helminth infections are soil transmitted parasites; thus, the host environment is important in determining exposure to and transmission of helminth parasites (2). In addition, the environment also plays a crucial role in determining variation in immune responses, pathogenesis of diseases as well as susceptibility versus resistance patterns during helminth infection. This can be the biotic environment of the individual which can constitutes the commensal microbial communities within the host or the other free living organismal life the host interacts within the environment including vectors and intermediate host for parasites. Other biotic factors including previous microbial experience and infection history of the host are important in influencing susceptibility and resistance to helminth infection (120–122). For example, primary infection activates memory $CD4^+$ T cells and alternatively activated macrophages that mediate resistance against secondary helminth infection (43, 123–125). There are also examples whereby previous helminth infection can make the host more susceptible to secondary helminth infection (126). Indeed, helminth co-infection may influence outcome of other infections and many studies have investigated the influence of previous helminth infections on the pathogenesis of other infectious diseases – including bacterial, protozoan, and viral diseases (120–122, 127–134). This experimental study design stems from the idea that mammals have co-evolved with helminth parasites and they could be part of the natural macrobiota of the host (135, 136). However, pre-existing disease and co-infections may also influence outcomes during helminth infection. For example, prior infection and co-infection of the protozoan parasite *Toxoplasma gondii* with the enteric nematode *H. polygyrus* can limit the host protective Type 2 immune response directed against the helminths making the host more susceptible to the worms (137). This results from the immune landscape in the host being skewed towards a Type 1 response by the protozoan parasite (137). A similar phenomenon has been reported wherein prior infection with protozoan parasites like *T. gondii* and *Plasmodium* parasites and viral pathogens like Human T-lymphotropic virus 1 (HTLV-1), which all induce a Type 1 immune response, can limit the host response during subsequent infection with helminth parasites like *Fasciola hepatica*, *Nippostrongylus brasiliensis* and *Strongyloides stercoralis* (122, 138–141). Hence, prior and co-infections by other pathogens can influence pathogenesis and susceptibility to helminths, thereby potentially contributing to inter-individual variations in immune response during helminth infection. In addition to biotic factors, these could also involve abiotic factors which constitute climatic factors such as the temperature, humidity, rainfall; physical factors such as the soil and mineral composition of the environment; and chemical factors such as the oxygen, nitrogen, and CO_2 levels in the environment. Factors such as this can influence the lifecycle of the parasite outside the host and therefore transmissibility of the helminth parasite. These biotic and abiotic factors can also have major implications on the tone of immune response as well as host susceptibility to helminth parasite infection thereby contributing to inter-individual variation during helminth infection (142).

Several studies have shown that the environmental differences can significantly influence the host immune phenotype and profile (143–146) and consequently susceptibility to subsequent helminth infections (147) (**Figure 1**). The importance of the role of environmental influences on the immune system can be appreciated from twin studies which show that variability in immune responses can be dictated in large part by acquired and not only genetic factors especially with increasing age emphasizing the influence of environmental factors on immune responses (148). Although, similar twin studies experiment in the context of helminth infection have not really been done in humans to understand the role of environment versus genetics during helminth infection. The use of mice models such as the rewilding mice model has helped us appreciate the critical role the host environment can play in outcomes during *T. muris* infection (147, 149). However, there is still a need to understand how the tissue microenvironment (3) and/or host macroenvironment (142, 150) can influence susceptibility patterns and helminth infection outcomes during exposure to various other helminth parasite types (hookworms, roundworms, tapeworms). New environmental mouse models such as "rewildings", "wildlings", "co-housing", "sequential infection models", "co-infection models", "chimeric wild mouse" etc (142, 146, 151–153) provide new opportunities to understand the role of environmental factors in influencing susceptibility to infection and re-infection by helminth parasite.

Rewilding mice involves introducing laboratory mice into an outdoor enclosure. This outdoor enclosure exposes mice to a natural environment including soil, weather, vegetation, microbial population, but protects against predation, and serves as a bridge between laboratory controlled experiments and what happens in a more natural environment (147). The rewilding mice model has already provided critical insights into the role of host macroenviroment on helminth infection outcomes (147, 149). Rewilded mice were more susceptible to the intestinal helminth parasite *T. muris* with higher worm burdens as well as more worm biomass than the laboratory controls (147). Rewilding experiments have also revealed how the environment contributes to the microbial diversity in the gut (149), provided insights into the role of fungal colonization to neutrophil circulation (143) as well as uncovered the role of environment and genetic factors in immune composition and responsiveness (144).

Besides rewilding, other approaches focus on increasing the microbial diversity in the gut environment, including fecal transplants, co-housing, exposure to dirty bedding (formites) and embryo transfer from wild and pet store mice (145, 146, 151, 153, 154). Thereby leading to various model including "wildlings", "chimeric lab-wild models", which have a natural and diverse metaorganisms at all body sites similar to wild and petstore mice (145, 146). These mice may have better translational value than specific pathogen free laboratory mice found in most biomedical centers with immune systems that are a better reflection of the human situation (142, 146, 152, 153).

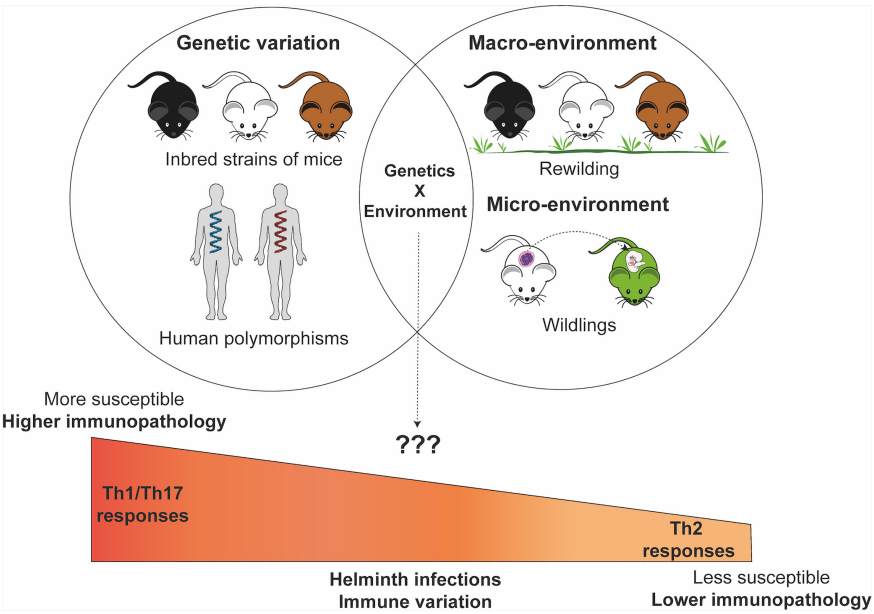

FIGURE 1 | Genetic and environmental factors contribute and interact to influence susceptibility patterns and pathogenesis of immune response during helminth infections. The Figure in this manuscript was created using images from the Servier Medical Art's image collection (smart.servier.com) and licensed under a Creative Commons Attribution 3.0.

These mice mount an immune response that limits exuberant responses which promotes survival following intense inflammatory conditions and are better able to control pathogen exposure compared to their SPF counterparts (145, 151). However in some cases, these mice generate stronger immune responses than their controls, for example, during exposure to house dust mite antigen (155). Other models have focused on sequentially infecting, co-infecting, persistently infecting SPF mice with various known microbial pathogens to expand their microbial experience, immunological landscape and microenvironment so that they better reflect the human experience (129, 156). This delivers a controlled set of pathogens to the mice and eliminates the requirement for special housing facility in order to use these mice to test various hypotheses in biomedical research.

In conclusion, there is considerable interest in developing these new tools (142, 152) to better model the human immune response in mice and to assess the role of environment and its interaction with genetic factors on susceptibility and resistance patterns during helminth infection.

The Microbiome as a Major Environmental Variable

The microbiome contributes immensely to an individual's biotic environment and could be an important environmental variable influencing outcomes to helminth infection. Since these microbial communities are found at epithelial barrier and mucosal surfaces where they can directly interact with helminth parasites, they can shape the tissue micro-environment niches and influence helminth infection outcomes (157–160). For example, the microbiome composition in mice prior to *H. polygyrus* infection or *S. mansoni* infection can influence the worm burden following helminth infection (157, 158). Similarly, oral colonization of mice with commensals such as *Lactobacillus casei*, *Bifidobacterium animalis* prior to helminth infection can alter the outcome of *T. muris* and *Strongyloides venezuelensis* infection in mice (161, 162). Furthermore, treatment of mice with *L. casei*, significantly increase the cecal worm burdens during *T. muris* infection while feeding mice with *B. animalis* significantly reduced *S. venezuelensis* worm burden and egg output (161, 162). There could be direct or indirect effects of these interactions. The effects of the microbiome composition on immune cell populations and epithelial cell function could influence infection outcomes (157, 163, 164). For example, the expression of *Pla2g1b*, an epithelial derived phospholipase A_2, that is also a host-derived anthelminthic factor is dependent on the intestinal microbiota (163). Similarly, the intestinal microbiome composition and abundance is associated with IL-10 signaling in the host (164). Sometimes, it is mechanistically unclear how microbiome changes can influence susceptibility and pathogenesis of disease during helminth infection, although studies have shown that helminth infection can influence the microbiome composition and diversity in the host recently reviewed here (159). A recent study by Moyat et al., 2022 using germ-free, antibiotic-treated, and specific pathogen-free mice clearly demonstrated that the intestinal bacteria composition can have an impact on host resistance to intestinal helminth *H. polygyrus* (165). Depletion of a complex microbiota through long term-treatment with antibiotics or in germ free mice resulted in more susceptibility to worm infection *via* a mechanism that is dependent on intestinal acetylcholine, a neurotransmitter,

necessary for intestinal motility (165). This suggests that the microbiome as a biotic factor, can influence the production of critical signaling molecules necessary for parasite clearance through the "weep and sweep" response. In contrast, *S. mansoni* infection requires a complex microbiome for greater parasite fecundity and pathology during infection (166, 167). Germ-free mice and antibiotic treated mice infected with *S. mansoni* infection resulted in decreased fecal egg counts as well as reduced intestinal pathology and inflammation (166, 167). In summary, the microbiome is a major biotic environmental factor that contributes to inter-individual variation during helminth infection through its effects on the immune and epithelial cell function of the host.

Other studies have suggested that the intestinal bacteria can directly influence parasite establishment, hatchability, and development (168–172). Various *in vitro* and *in vivo* methods demonstrated that the presence of *Escherichia coli*, a common intestinal commensal (173), is important for hatchability of *T. muris* eggs. The role of *E. coli* in parasite establishment is driven by the presence of Type 1 Fimbriae as well as release of microbial byproducts (168, 169). Differences in egg hatching following infection could affect the establishment of the parasite and subsequently the parasite burden, hence the presence or absence of specific commensal bacteria may directly influence parasite burden independently of the host immune response. Experiments with germ free mice or gnotobiotic animals in helminth infection models also demonstrate that the success of parasite infection and fitness is dependent on presence of commensal bacteria (174–178). Therefore, differences in abundance and composition of intestinal bacteria, which could be influenced by use of antibiotics, could contribute to inter-individual variation in parasite burden observed in population studies during helminth infection.

Additionally, the contribution and role of many other components of the microbiome such as fungi, viruses, and archaea (179) in host immune response and parasite development remains poorly understood and whether these components also influence susceptibility and disease pathogenesis during helminth infection needs to be further explored.

INTERPLAY BETWEEN ENVIRONMENT AND HOST GENETICS

While host genetics are important contributors towards response to helminth infections (102). Twin studies suggest that there are both heritable and non-heritable explanations for variation in immune responses (148). However, these two critical factors rarely exist in isolation (142, 180). There are limited studies that assess the additive and interactive effect of environmental and genetic effect on immune responses. As individuals with similar genetic profiles would usually live within the same communities and environment, it is challenging to dissociate one from the other in human studies.

Therefore, mouse models could be helpful in dissociating the complex interactions that exist between genetic and environmental contributors to variation in helminth infection (**Figure 1**). For example, there are indicators that genetic differences in parasite resistance in the laboratory setting may be lost in a natural environment. While *H. polygyrus* infection of BALB/c and C57BL/6 mice in a laboratory setting shows clear differences in resistance to infection, natural infection results in no observable significant differences between these two inbred resistant and susceptible mice (181). Although mechanistically unexplained, a subsequent study suggests that the chronicity of the infection model could explain why the difference was lost between these two different inbred strains of mice (182). In our own studies, differences in susceptibility between wild-type mice and susceptible STAT6$^{-/-}$ knockout mice to *T. muris* infection in the lab setting are no longer observed when these mice are were placed in the re-wilded environment and infected with *T. muris* (147).

Such interactions between genetics and the environment could be complex and mechanistically distinct. As described above, genetic factors such as MHC haplotype may indirectly influence microbial communities within the host (biotic environmental factors) to alter susceptibility to helminth infection (79). How the environmental pressures influences the heritability of helminth resistance genes in the population is also of interest, as shown in a study in sheep whereby significant genotype by environment interactions persists following infection with another helminth parasites (183).

Other host variables such as age, sex, nutritional status could interact with genetics and environmental factors to influence immunity and affect the outcome of helminth infection. A combination of controlled re-wilding experiments (142–144, 147) and perhaps twin studies in helminth endemic populations with detailed questionnaires may provide further insights into the dynamics of interactions of such complex factors.

DISCUSSIONS AND CONCLUSIONS

Environmental exposure and host genetic background are important drivers of inter-individual variation in susceptibility and outcome of helminth parasite infections. Both play a role in driving heterogeneity of responses either as independent variables or through specific interactions that remain poorly understood. Since disease morbidity and parasite burden is observed primarily in small subsets of infected individuals, it is important that necessary resources and research efforts are allocated into studies deciphering how genetic and environmental interactions influences susceptibility to the world's most neglected disease in human and veterinary medicine. This will require bringing together skillsets and technologies from diverse fields including ecology, quantitative genetics, genomics, immunology, biostatistics, and parasitology. Together, such studies can improve our understanding of key translational factors that regulate immune responses during parasitic helminth infection. Findings from a diverse range of inbred and outbred strains of mice in different environments might provide a more accurate reflection of factors important in diverse human and animal population under free living conditions. There are also opportunities for the identification of new pathways and alleles or regulatory elements that regulate responses in other Type 2 immune mediated diseases such as allergic, metabolic and

fibrotic diseases (3, 88). Additionally, there is a need to address the rising incidence of drug resistance seen in the use of anti-helminthics in human and veterinary medicine (184, 185). To further complete this picture, exciting new studies are beginning to show the importance of heterogeneity in helminth parasite genetic factors in the susceptibility and resistance patterns during helminth infection (105–107). The role of parasite genetic diversity in the pathogenesis and outcome of helminth infection, remains a relatively understudied and interesting area to investigate. Parasite genetic heterogeneity could influence the excretory/secretory products produced from these parasites and influence the immunomodulatory properties of these factors. How this might contribute to inter-individual variation to helminth infection is an interesting area to explore.

AUTHORS CONTRIBUTIONS

OO wrote the first draft of the manuscript. CS and P'NL made significant direct and intellectual contribution to the manuscript. CS made the figure. P'NL edited the manuscript. All authors contributed to the article, read, and approved the submitted version.

FUNDING

This work was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH Order #NLR2110677 and funded the open access publication fees.

ACKNOWLEDGMENTS

The authors apologize to those authors whose exemplary work we are unable to cite. The Figure in this manuscript was created using images from the Servier Medical Art's image collection (smart.servier.com) and licensed under a Creative Commons Attribution 3.0.

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ORIGINAL RESEARCH
published: 02 June 2022
doi: 10.3389/fimmu.2022.809264

A Molecular Analysis of Memory B Cell and Antibody Responses Against *Plasmodium falciparum* Merozoite Surface Protein 1 in Children and Adults From Uganda

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Edited by:
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Reviewed by:
D. Herbert Opi,
Burnet Institute, Australia
Anna Färnert,
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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: 04 November 2021
Accepted: 05 May 2022
Published: 02 June 2022

Citation:
Gonzales SJ, Clarke KN, Batugedara G, Garza R, Braddom AE, Reyes RA, Ssewanyana I, Garrison KC, Ippolito GC, Greenhouse B, Bol S and Bunnik EM (2022) A Molecular Analysis of Memory B Cell and Antibody Responses Against Plasmodium falciparum Merozoite Surface Protein 1 in Children and Adults From Uganda. Front. Immunol. 13:809264. doi: 10.3389/fimmu.2022.809264

Memory B cells (MBCs) and plasma antibodies against *Plasmodium falciparum* (*Pf*) merozoite antigens are important components of the protective immune response against malaria. To gain understanding of how responses against *Pf* develop in these two arms of the humoral immune system, we evaluated MBC and antibody responses against the most abundant merozoite antigen, full-length *Pf* merozoite surface protein 1 ($PfMSP1_{FL}$), in individuals from a region in Uganda with high *Pf* transmission. Our results showed that $PfMSP1_{FL}$-specific B cells in adults with immunological protection against malaria were predominantly IgG^+ classical MBCs, while children with incomplete protection mainly harbored IgM^+ $PfMSP1_{FL}$-specific classical MBCs. In contrast, anti-$PfMSP1_{FL}$ plasma IgM reactivity was minimal in both children and adults. Instead, both groups showed high plasma IgG reactivity against $PfMSP1_{FL}$, with broadening of the response against non-3D7 strains in adults. The B cell receptors encoded by $PfMSP1_{FL}$-specific IgG^+ MBCs carried high levels of amino acid substitutions and recognized relatively conserved epitopes on the highly variable PfMSP1 protein. Proteomics analysis of $PfMSP1_{19}$-specific IgG in plasma of an adult revealed a limited repertoire of anti-MSP1 antibodies, most of which were IgG_1 or IgG_3. Similar to B cell receptors of $PfMSP1_{FL}$-specific MBCs, anti-$PfMSP1_{19}$ IgGs had high levels of amino acid substitutions and their sequences were predominantly found in classical MBCs, not atypical MBCs. Collectively, these results showed evolution of the PfMSP1-specific humoral immune response with cumulative *Pf* exposure, with a shift from IgM^+ to IgG^+ B cell memory, diversification of B cells from germline, and stronger recognition of PfMSP1 variants by the plasma IgG repertoire.

Keywords: malaria, adaptive immune response, humoral immunity, antibodies, memory B cells, IgM, IgG, somatic hypermutation

INTRODUCTION

Malaria, caused by the parasite *Plasmodium falciparum* (*Pf*), is responsible for more than half a million deaths every year, of which two-thirds occur in children under the age of five (1). A much larger number of individuals experience non-fatal malaria, amounting to an estimated 241 million cases of disease in 2020. Although the mortality rate for malaria has slowly but consistently declined over the past two decades, the decrease in malaria incidence has plateaued in the past five years. Current interventions are thus insufficient for malaria elimination and novel tools, such as a highly efficacious malaria vaccine, are urgently needed in the fight against this devastating disease. RTS, S, the only malaria vaccine that has elicited protection against (severe) malaria in a phase III clinical trial, had an efficacy against clinical malaria of 30 – 40% in infants and young children (2). Vaccine efficacy was high shortly after vaccination but declined rapidly, and was lower against parasites that were genetically different from strain 3D7 that the subunit vaccine RTS,S was based on (3). Compared to vaccination, repeated natural *Pf* infections eventually elicit superior immunity, consisting of relatively long-lived antibody responses (~2 – 4 years) with cross-strain reactivity (4, 5). This naturally acquired humoral immunity against malaria is associated with the presence of circulating immunoglobulin G (IgG) against *Plasmodium* blood stage antigens (6–13). Recently, immunoglobulin M (IgM) has received increased attention as IgM responses were also shown to correlate with protection and were able to inhibit parasite growth *in vitro* (14–16). A complete understanding of the development of humoral immune responses against *Pf* blood stage antigens over time will help with the development of a more effective and longer lasting vaccine than RTS,S.

Effective and long-lasting humoral immune responses against pathogens consist of long-lived memory B cells (MBCs) in the circulation and plasma cells that reside in the bone marrow and secrete antibody into the circulation [reviewed in (17)]. In the case of *Pf*, long-lived humoral immunity develops over the course of years of repetitive infections, leaving young children susceptible to disease. With cumulative *Pf* exposure, the quality of B cell responses gradually improves. Specifically, the abundance of parasite-specific B cells increases over time (18). In addition, the longevity of plasma cells generated in response to *Pf* infection improves with subsequent exposures (4, 19). Moreover, both MBC and antibody responses gradually broaden to target a larger number of parasite antigens in adolescents and adults (19, 20). Finally, recurrent infections drive the generation of antibodies capable of mediating cross-strain immunity, which is strongly associated with protection (5). Most studies of the development of antibody responses against *Pf* have been performed using serum or plasma, thus precluding the investigation of molecular characteristics of *Pf* -specific antibodies. In addition, little is known about the connection between the MBC and plasma cell compartments. This information would be useful for understanding how durable immunity against malaria develops and may enable us to harness lessons from naturally acquired immunity for improved vaccine design.

In this study, we set out to investigate differences in humoral immune responses between children with incomplete protection against malaria and adults who have developed strong immunological protection, to better understand how MBC and antibody responses develop over the course of life-long *Pf* exposure. To do this, we compared the antibody and MBC response against *Pf* antigen merozoite surface protein 1 (PfMSP1) between children and adults living in a region of high *Pf* transmission in Uganda. PfMSP1 is a large, polymorphic, and highly immunogenic protein expressed ubiquitously on the surface of the parasite during the late schizont and merozoite stages (21, 22). PfMSP1 has long been considered a vaccine target since antibody responses against this protein have been associated with protection (6, 21, 23), although results from PfMSP1-based vaccine trials have been disappointing (24, 25). However, since PfMSP1 has high antigenic heterogeneity across *Pf* strains (26, 27), it is a model antigen well-suited to assess antibody cross-strain reactivity elicited by natural infection. In addition, PfMSP1 is commonly used to study *Plasmodium*-specific MBCs in both humans (15, 16, 28, 29) and mice (30–32), and this study therefore adds to a growing body of literature on PfMSP1-specific B cell responses. We studied the isotype of PfMSP1-specific MBCs, characteristics of monoclonal B cell receptor sequences isolated from IgM^+ and IgG^+ MBCs, cross-strain reactivity against PfMSP1 variants in both plasma antibodies and B cells, and plasma IgM and IgG reactivity against PfMSP1 and other merozoite antigens (**Figure 1**). In addition, we analyzed the anti-PfMSP1 plasma IgG repertoire and investigated characteristics of antibodies that were found in both the MBC and plasma IgG compartment in more detail.

RESULTS

Study Population

To study differences in naturally acquired B cell responses against PfMSP1 between partially immune and immune individuals, we selected children (on average 6.3 years of age, range 4 – 8) and adults (on average 42 years of age, range 22 – 72) residing in Tororo, Uganda, a region with high malaria transmission intensity year-round (**Table 1** and **Supplementary Table 1**) (33). These age ranges were chosen based on pre-existing data about development of immunological protection to malaria for people living in this region (see also Materials and Methods). These individuals were participants of cohort studies, with the exception of two Ugandan adults who were anonymous blood donors. The adult cohort participants had developed immunity against disease, defined as the absence of a malaria episode in the year preceding or following sample collection. All children had at least one febrile malaria

Abbreviations: BCR, B cell receptor; HCDR3, heavy chain complementarity determining region 3; Ig, immunoglobulin; MBC, memory B cell; mAb, monoclonal antibody.

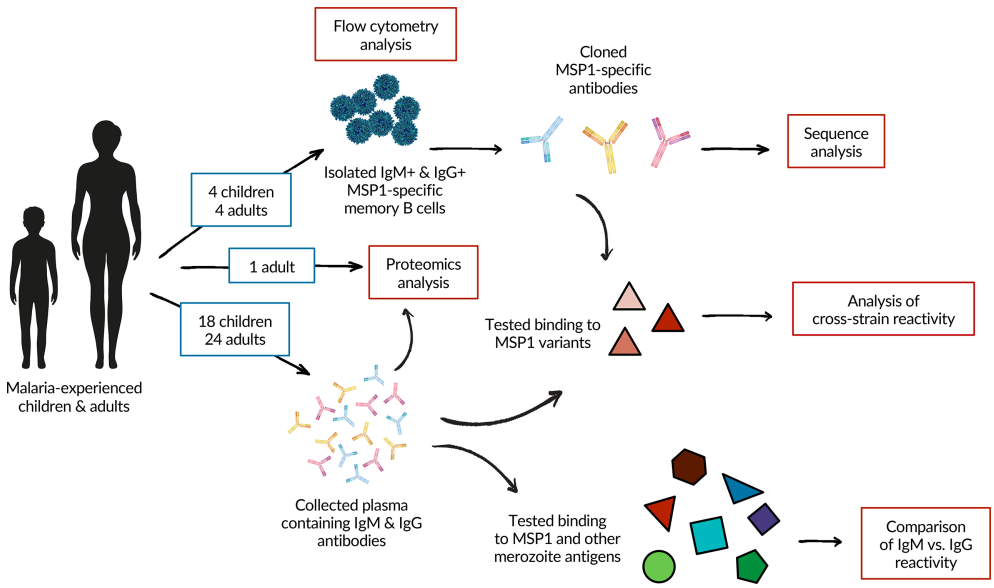

FIGURE 1 | A schematic overview of the experimental approach and analyses performed in this study. A total of 26 adults and 20 children were included in the study. Samples from four children and four adults were used for flow cytometric analysis and the isolation of PfMSP1-specific B cells. Two individuals from each group were also included in the analysis of plasma IgM and IgG responses against PfMSP1 and other merozoite antigens. See **Supplementary Table 1** for details on which donors were included in which experiments.

episode in the year preceding or following sample collection, but in that time frame also had at least one additional *Pf* infection (either microscopic or submicroscopic) detected during passive surveillance that was cleared in the absence of antimalarial treatment, collectively defined as partial immunity (**Table 1** and **Supplementary Table 1**). All adults and most children were blood smear negative at the time of sample collection, while a considerable proportion of individuals had subpatent levels of parasitemia, with no difference in prevalence between adults and children (**Table 1**). We did not have access to medical information for the two anonymous adult donors, and we included them in the study based on their high levels of plasma antibodies against PfGLURP-R2, PfAMA1, and the C-terminal 19 kDa fragment of PfMSP1 ($PfMSP1_{19}$), suggesting frequent exposure to *Pf* at levels equal to that of the adult cohort participants (**Supplementary Figure 1**).

PfMSP1-Specific B Cells Have a Classical Phenotype and Are Enriched for IgG in Adults

For four children and four adults, cryopreserved PBMCs were used to first isolate bulk B cells, followed by staining with fluorescently labeled tetramers of full-length PfMSP1 from *Pf* strain 3D7 ($PfMSP1_{FL\text{-}3D7}$) and decoy tetramers of the irrelevant protein rat CD200 for analysis by flow cytometry, a strategy developed and used by others (30, 34) (**Figure 2A**, second panel). In this approach, $PfMSP1_{FL\text{-}3D7}$ tetramers are conjugated to a single fluorochrome (PE), while the decoy tetramers are dual-labeled with PE and AF647 to allow discrimination between $PfMSP1_{FL\text{-}3D7}$-specific B cells (PE^+AF647^-) and B cells that bind other parts of the tetramer (PE^+AF647^+). In both children and adults, $PfMSP1_{FL\text{-}3D7}$-specific B cells (defined as $CD19^+$ $CD20^+$ $PfMSP1^+_{FL\text{-}3D7}decoy^-$) were predominantly found among $CD21^+CD27^+$ classical memory B cells (MBCs) (**Figure 2B**). This was also observed when analyzing IgM^+ and IgG^+ B cells separately (**Supplementary Figure 2**). In addition, in both groups, very few $PfMSP1_{FL\text{-}3D7}$-specific B cells were found among $CD21^-CD27^-$ atypical MBCs (**Figure 2B**). To further define the phenotype of $PfMSP1_{FL\text{-}3D7}$-specific B cells, we determined the isotype usage of $PfMSP1_{FL\text{-}3D7}$-specific classical MBCs. Children showed much larger percentages of IgM^+ (median, ~70%) than IgG^+ (median, ~10%) classical MBCs in the total repertoire, which was reflected in the percentage of IgM^+ and IgG^+ PfMSP1-specific classical MBCs (**Figure 2C**). In contrast, IgM^+ and IgG^+ classical MBCs were equally abundant in adults in the total repertoire, while IgM^+ PfMSP1-specific classical MBCs were depleted and IgG^+ $PfMSP1_{FL\text{-}3D7}$-specific classical MBCs were slightly enriched (**Figure 2C**). The ratio of IgG^+ to IgM^+ $PfMSP1_{FL\text{-}3D7}$-specific classical MBCs was higher in all adults as compared to the children (**Figure 2D**). Collectively, these results suggest that the B cell response against PfMSP1 is dominated by IgM^+ classical MBCs in children, while it is dominated by IgG^+ classical MBCs in adults. These results are in line with a recent report that showed an increased frequency of IgM usage among *Pf*-specific B cells in young children as compared to older children and adults in Mali, where malaria transmission is highly seasonal (16), suggesting that this is a general feature of the B cell response to *Pf* in children in malaria-endemic regions.

TABLE 1 | Demographic and clinical characteristics of study participants.

	Adult	Children	P value[b]
Number of Subjects	26[a]	20[a]	
Age (yrs; Median, range)	41 (22 – 72)	6.3 (4.8 – 8.2)	<0.0001
Sex (n, %)			0.009
Male	3 (13%)	10 (50%)	
Female	21 (87%)	10 (50%)	
G6PD genotype (n, %)			0.77
Wild type	13 (57%)	12 (67%)	
Homozygote	1 (4%)	0 (0%)	
Heterozygote	7 (30%)	5 (28%)	
Hemizygote	2 (9%)	1 (6%)	
Alpha-thalassemia genotype (n, %)			0.23
Wild type	11 (48%)	8 (44%)	
Alpha +	9 (39%)	10 (56%)	
Alpha 0	3 (13%)	0 (0%)	
HbS genotype (n, %)			0.20
Wild type	18 (77%)	17 (94%)	
Homozygote	0 (0%)	0 (0%)	
Heterozygote	5 (23%)	1 (6%)	
CD36 genotype (n, %)			0.43
Wild type	18 (74%)	16 (89%)	
Homozygote	0 (0%)	0 (0%)	
Heterozygote	6 (26%)	2 (11%)	
Malaria diagnosis at sampling (n, % positive)	0 (0%)	0 (0%)	>0.99
***Plasmodium* asexual stages by microscopy at sampling** (n, %)	0 (0%)	5 (25%)	0.01
Submicroscopic *Plasmodium*, by LAMP at sampling (n, %)	12 (50%)	3 (38%)	0.70
Malaria diagnosis within one year of sampling date (n, %)	0 (0%)	20 (100%)	<0.0001
Parasitemia cleared without treatment within one year of sampling date (n, %)	23 (96%)	20 (100%)	>0.99

[a]*Sample size within demographic and disease parameters may be less than overall sample size due to missing data, see* ***Supplementary Table 1****.*

[b]*All P values were obtained using a Fisher's exact test, except for age. Differences in age between the two groups were tested using an unpaired Student's t-test.*

Yrs, years; G6PD, Glucose-6-phosphate dehydrogenase; LAMP, loop-mediated isothermal amplification method.

Recombinant Antibodies Isolated From Memory B Cells Are PfMSP1-Specific and Inhibit Parasite Growth

To confirm antigen-specificity of $PfMSP1_{FL\text{-}3D7}$-specific B cells, IgM^+ and IgG^+ $PfMSP1_{FL\text{-}3D7}$-specific B cells were single-cell-sorted into 96-well culture plates containing CD40L-expressing feeder cells, IL-2, IL-21, the TLR-9 agonist ODN2006, and transferrin, which collectively promote B cell survival, expansion, and differentiation into antibody-secreting cells (35, 36). This allowed us to screen B cell clones for antigen-specificity prior to cloning and expression of recombinant mAbs. B cell supernatants were tested for the presence of anti-$PfMSP1_{FL\text{-}3D7}$ antibodies by a *Pf* strain 3D7 merozoite ELISA or Luminex assay using recombinant $PfMSP1_{FL\text{-}3D7}$. These assays were implemented into our analysis pipeline only in later experiments and these data are therefore missing for samples from donors 170 and 2 as these were analyzed earlier. The variable regions of antibodies with confirmed reactivity to merozoites or recombinant $PfMSP1_{FL\text{-}3D7}$ were cloned into linear expression cassettes, expressed as recombinant IgG_1, and again tested for $PfMSP1_{FL\text{-}3D7}$ reactivity by Luminex (**Figure 3A**). In total, we isolated 31 mAbs with reactivity to $PfMSP1_{FL\text{-}3D7}$ derived from three children and three adults.

As was reported in a recently published article (15), most anti-$PfMSP1_{FL\text{-}3D7}$ IgM antibodies lost their reactivity to $PfMSP1_{FL\text{-}3D7}$ when expressed as IgG_1, which is likely a result of the lower avidity of monomeric IgG as compared to pentameric IgM. We therefore cloned a multimerization domain into the C-terminus of the IgG_1 heavy chain to express IgM-derived antibodies as pentameric IgG. In contrast to results reported by Thouvenel et al. (15), we were unable to rescue the $PfMSP1_{FL\text{-}3D7}$-reactivity of IgM-derived variable regions by expression as pentameric IgG, possibly because we tested only a small number of IgM-derived antibodies (**Figure 3A**). The only pentameric IgG with reactivity to $PfMSP1_{FL\text{-}3D7}$ in the Luminex assay was also reactive when expressed in monomeric form. To further confirm the specificity and functionality of the isolated anti-PfMSP1 mAbs, we performed immunofluorescence assays on segmented schizonts for three randomly selected IgG mAbs, showing the expected surface staining of merozoites (**Figure 3B**). We also performed a growth-inhibition assay using blood stage *Pf* (as decribed in (37) with modifications, see Material & Methods) for six IgG mAbs that were selected based on high expression levels in culture. As a negative control, we included antibody elution buffer (100 mM glycine-HCl, pH 2.7) that was buffer exchanged to PBS alongside purified antibodies. mAbs were tested at a single concentration of 200 μg/ml, which is on the low end of what is commonly used to test growth-inhibitory activity of mAbs. The six mAbs showed on average 51% (range, 17% – 65%) inhibition of *Pf* growth as compared to the negative control, demonstrating their ability to inhibit parasite replication (**Figure 3C**).

PfMSP1-Specific IgG^+ MBC Lineages From a Malaria-Experienced Adult Are Highly Diversified and Expanded

Next, we set out to study the molecular characteristics of antibodies encoded by $PfMSP1_{FL\text{-}3D7}$-specific MBCs. In total, we isolated 6 IgM and 25 IgG mAbs with confirmed $PfMSP1_{FL\text{-}3D7}$ reactivity from 6 donors. Full-length heavy and light chain variable regions were obtained and analyzed using IMGT/V-QUEST (38) to determine the level of amino acid substitutions in the V gene segments of the variable regions. $PfMSP1_{FL\text{-}3D7}$-specific IgM^+ MBCs from adults and children carried few amino acid substitutions in both the heavy and light chain V gene segments (average, <1% amino acid changes), while many of the IgG^+ MBCs were highly mutated (>15% amino acid changes; **Figures 4A, B**). None of the IgM^+ MBCs were clonally related to each other, defined as sequences using the same heavy chain V and J gene and having a highly similar heavy chain complementarity determining region 3 (HCDR3; ≥85% amino acid similarity), which is likely a result of the very small number of IgM sequences obtained. In contrast, several clonally expanded IgG^+ MBC lineages were identified in adult donor 2, from whom most mAbs were derived (**Figures 4A, B**). B cells

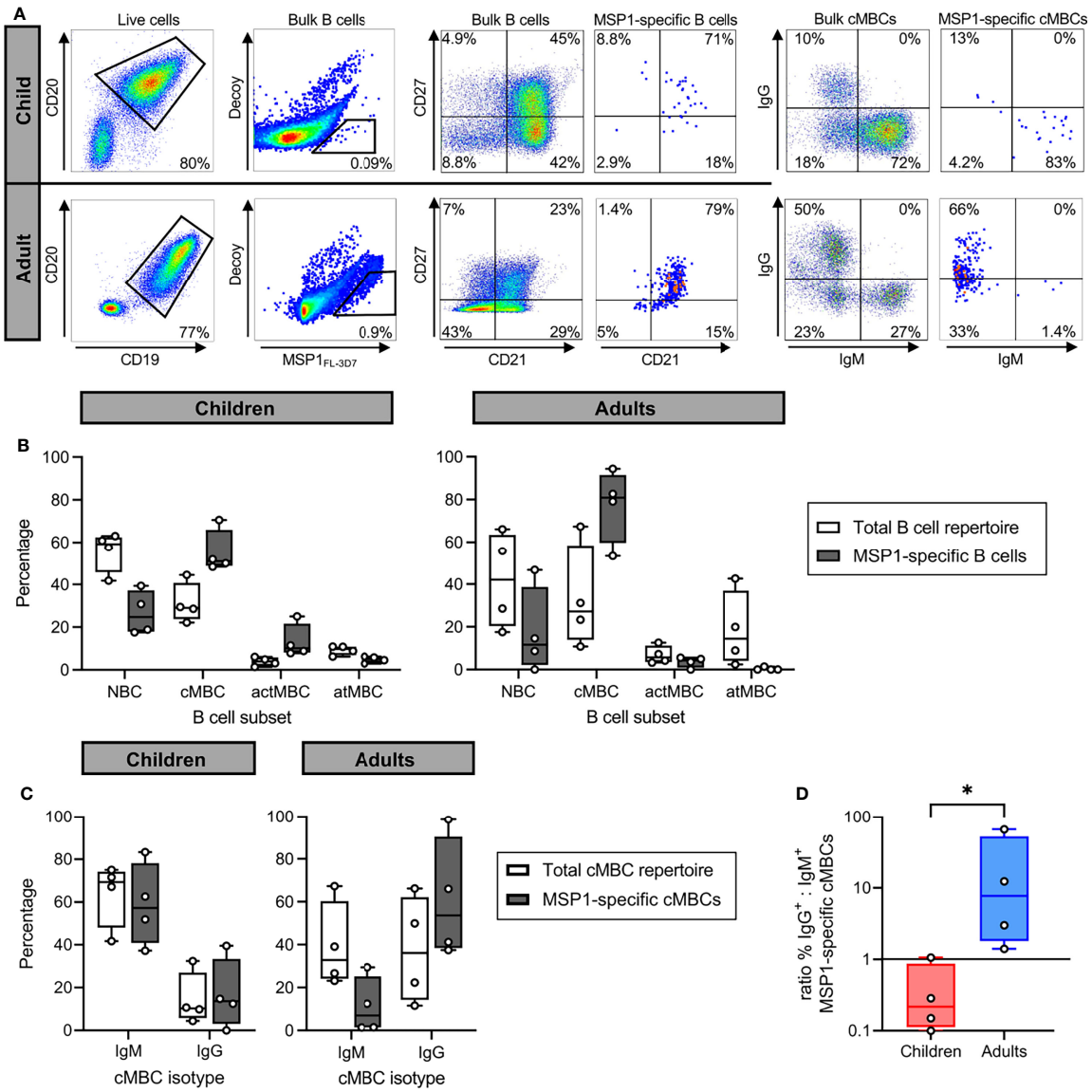

FIGURE 2 | The isotype of PfMSP1$_{FL\text{-}3D7}$-specific B cells in malaria-experienced children and adults. **(A)** Representative flow cytometry gating for the sorting and analysis of bulk B cells and PfMSP1$_{FL\text{-}3D7}$-specific B cells. B cells were first gated as live CD19^{+}CD20^{+} cells, followed by sorting of *PfMSP1*$^{\pm}_{FL\text{-}3D7}$*decoy*$^{-}$ cells. The four major B cell subsets are defined as follows: naïve B cells (NBC), CD21^{+} CD27^{-}; classical memory B cells (cMBC), CD21^{+} CD27^{+}; activated memory B cells (actMBC), CD21^{-} CD27^{+}; and atypical memory B cells (atMBC), CD21^{-} CD27^{-}. Data are shown as pseudocolor plots, in which overlapping cells in the plots showing PfMSP1$_{FL\text{-}3D7}$-specific B cells and cMBCs are shown in orange and red. **(B)** Relative abundance of major B cell subsets among the total B cell repertoire and among PfMSP1$_{FL\text{-}3D7}$-specific B cells in partially immune children (n = 4) and immune adults (n = 4). **(C)** The percentage of IgM^{+} and IgG^{+} B cells among the total repertoire of cMBCs and among PfMSP1$_{FL\text{-}3D7}$-specific cMBCs in malaria-experienced children (n = 4) and adults (n = 4). In panels B and C, differences were not tested for statistical significance because the appropriate non-parametric test for paired data (Wilcoxon signed-rank test) does not return a P value lower than 0.13 when using groups of 4. **(D)** The ratio of the percentage of IgG^{+} over IgM^{+} PfMSP1$_{FL\text{-}3D7}$-specific cMBCs in malaria-experienced children and adults. A data point with value 0 was plotted at 0.1 for visualization purposes. The difference between groups was tested for statistical significance using a Mann Whitney test. *P < 0.05.

belonging to expanded lineages were among those that were the most diversified from the germline antibody sequences, particularly in the heavy chain V gene segment. Finally, several IgG sequences harbored long HCDR3s (≥ 20 amino acids), while IgM sequences had average or relatively short HCDR3s (**Figure 4C**).

Recombinant mAbs Isolated From Memory B Cells Recognize Relatively Conserved Epitopes on PfMSP1

A study of B cell responses upon influenza virus vaccination reported that the largest clonal B cell families were directed against the most conserved epitopes (39). Based on these

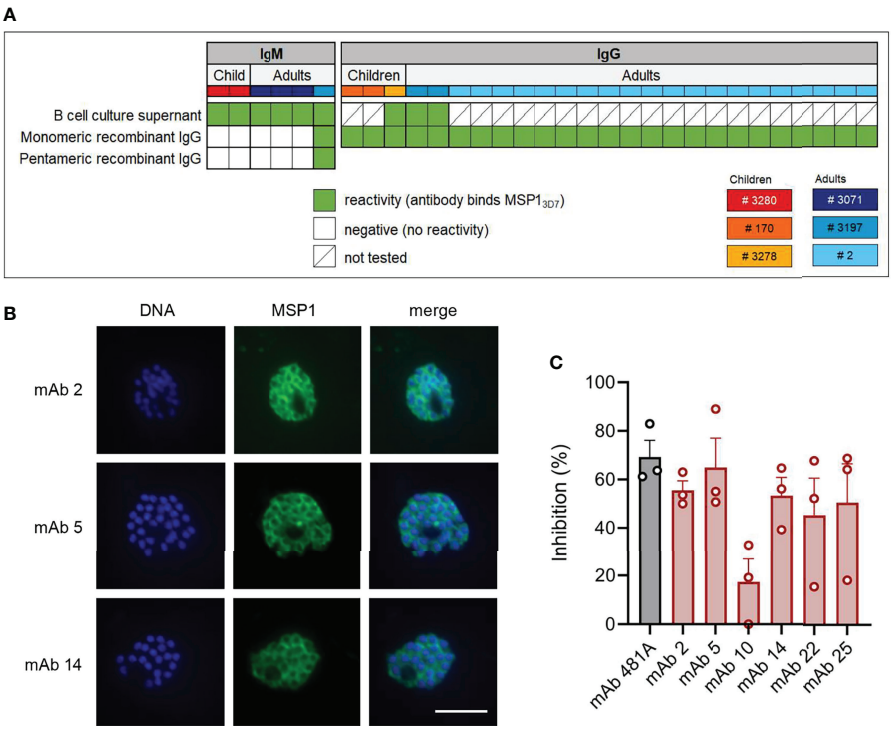

FIGURE 3 | PfMSP1$_{FL\text{-}3D7}$ reactivity and growth inhibitory activity of anti-PfMSP1 monoclonal antibodies isolated from memory B cells. **(A)** PfMSP1$_{FL\text{-}3D7}$ reactivity of supernatants of clonal MBC cultures determined by ELISA or Luminex assay and reactivity of mAbs obtained from MBCs (recombinant IgG) as determined by Luminex assay. The donor from whom mAbs were isolated is color-coded in the top row. Recombinant mAbs that were derived from IgM$^+$ MBCs were tested as both monomeric and pentameric recombinant IgG. **(B)** Immunofluorescence imaging of segmented schizonts using three select mAbs isolated from MBCs of adult donor 2. At this stage, parasites consist of 16 – 32 merozoites, each with its own nucleus (shown in blue), and express PfMSP1 on the parasite cell surface (shown in green). As expected, the overlay shows that PfMSP1 does not colocalize with the nucleus, but instead forms a circular pattern around each individual merozoite. Representative images of two experiments are shown. Scale bar is 5 µm. **(C)** *P. falciparum* growth inhibition by six select mAbs isolated from adult donor 2. Inhibition was calculated relative to a negative control (antibody elution buffer that was buffer exchanged to PBS alongside purified antibodies). Results shown are the average + SEM from three independent experiments. mAb 481A, an anti-PfAMA1 mAb with confirmed growth-inhibitory activity, served as a positive control.

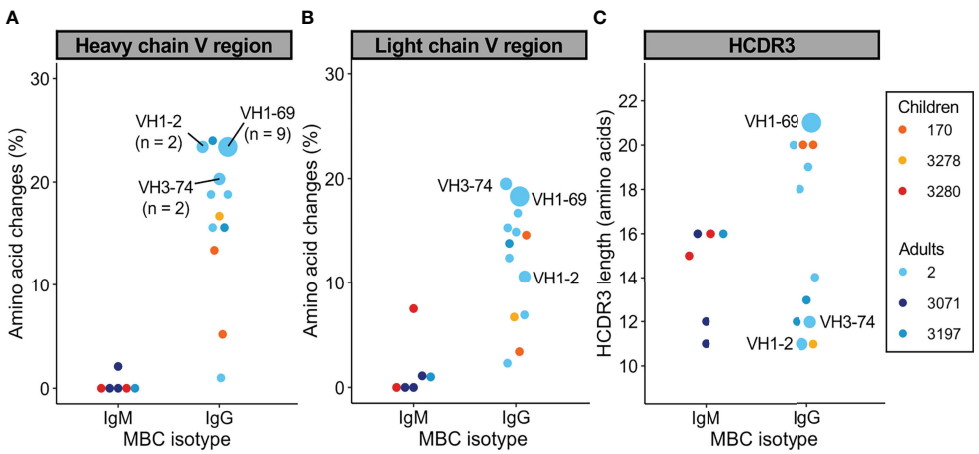

FIGURE 4 | Sequence analysis of the variable heavy and light chains of PfMSP1$_{FL\text{-}3D7}$-specific memory B cells. **(A, B)** Percentage of amino acid changes in the V gene segments of the heavy chain **(A)** and light chain **(B)** variable regions. **(C)** Length of HCDR3s. In all graphs, sequences from expanded clonal lineages were grouped. The VH gene usage and size of expanded clonal lineages are indicated.

observations, we hypothesized that clonally expanded anti-$PfMSP1_{FL\text{-}3D7}$ IgG^+ MBC lineages would have cross-strain reactivity, whereas non-expanded IgG^+ MBC lineages would be strain-specific. To test this hypothesis, we developed a Luminex assay using beads coated with full-length PfMSP1 variants from the geographically and genetically distinct *Pf* strains 3D7 (African origin; $PfMSP1_{FL\text{-}3D7}$), Dd2 (South-east Asian origin; $PfMSP1_{FL\text{-}Dd2}$), and HB3 (Central American origin; $PfMSP1_{FL\text{-}HB3}$). $PfMSP1_{FL\text{-}3D7}$ and $PfMSP1_{FL\text{-}Dd2}$ only differ in the sequence of block2, which is the most polymorphic region of PfMSP1, highly immunogenic, and associated with protective antibody responses (40–42), while $PfMSP1_{FL\text{-}HB3}$ is divergent throughout most of the protein (**Supplementary Figure 3**). To confirm that measuring reactivity against these PfMSP1 variants in a multiplex format does not lead to unexpected results, we compared the reactivity of several mAbs against these PfMSP1 variants in a multiplex assay versus singleplex assay, yielding a strong correlation between the results (**Supplementary Figure 4**). We then tested 22 IgG^+ B cell-derived mAbs for binding to the three PfMSP1 variants, while 3 mAbs were excluded due to antibody expression issues. 21 out of 22 mAbs (95%) tested in this assay bound to $PfMSP1_{FL\text{-}3D7}$ (**Figure 5A**). This high reactivity with $PfMSP1_{FL\text{-}3D7}$ was expected since this protein was used for the isolation of $PfMSP1_{FL\text{-}3D7}$-specific B cells. It is therefore unclear why one mAb bound $PfMSP1_{FL\text{-}Dd2}$ but not $PfMSP1_{FL\text{-}3D7}$. All other mAbs showed reactivity with at least one other PfMSP1 variant, and the majority showed reactivity with both $PfMSP1_{FL\text{-}Dd2}$ and $PfMSP1_{FL\text{-}HB3}$, including all mAbs from the largest clonal lineage (**Figure 5A**). In contrast to our hypothesis, no obvious difference in breadth of reactivity was observed between the single mAb clones and the expanded mAb lineages. These results suggest that the majority mAbs recognized relatively conserved epitopes, irrespective of the level of clonal expansion of the B cell lineage. Unfortunately,

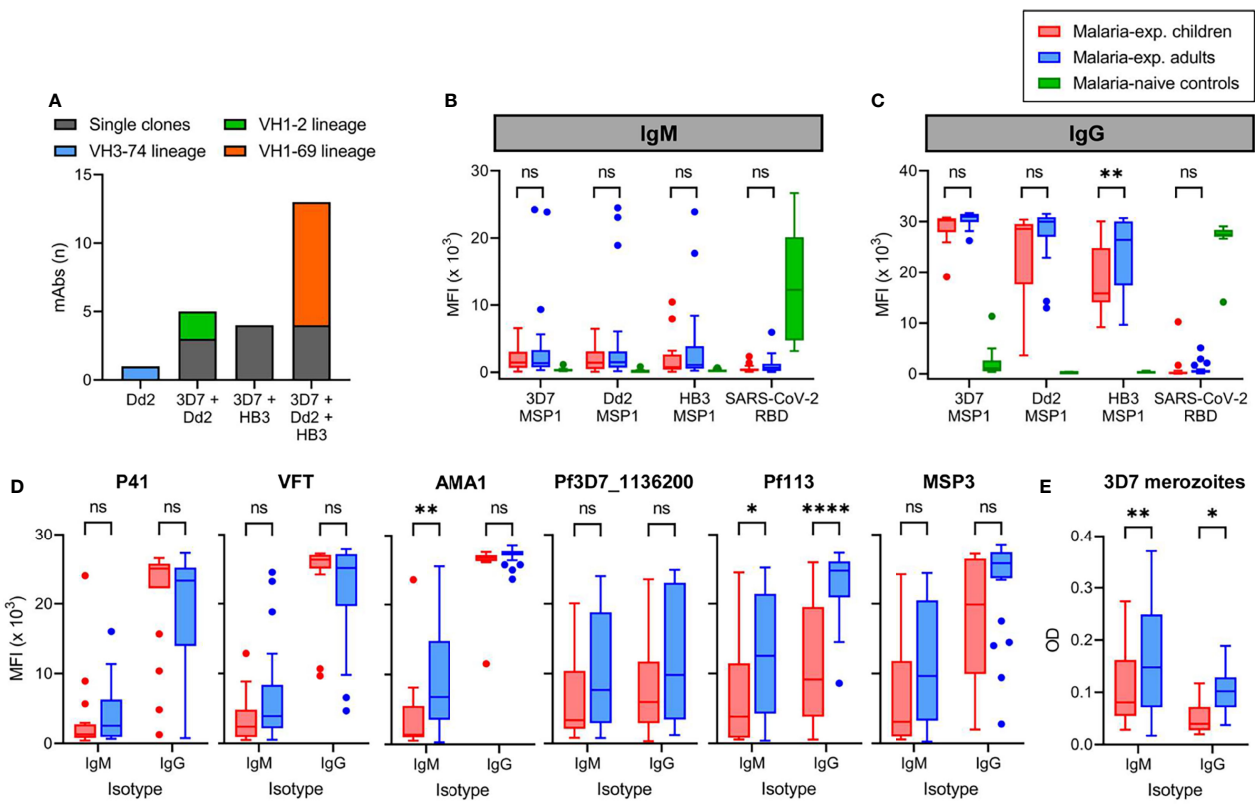

FIGURE 5 | Reactivity of monoclonal antibodies and plasma IgM and IgG against different strains of *P. falciparum*. **(A)** The number of anti-PfMSP1 recombinant monoclonal antibodies (mAbs) with reactivity against recombinant full-length PfMSP1 of three different *P. falciparum* strains in a Luminex assay. Each mAb is color-coded based on whether it was a single clone or derived from an expanded clonal B cell lineages. The VH3-74 lineage consisted of two MBCs, of which we were unable to retrieve the light chain for the second mAb. For this reason, only a single mAb of this lineage was tested here. **(B, C)** Reactivity of plasma IgM **(B)** and IgG **(C)** from malaria-experienced children (n = 18) and adults (n = 24), and malaria-naive recovered US COVID-19 patients (control; n = 10) against recombinant full-length PfMSP1 of *P. falciparum* strains 3D7, Dd2, and HB3, as well as SARS-CoV-2 receptor binding domain (RBD), as determined by Luminex assay. Values shown are mean fluorescence intensity (MFI). **(D)** Reactivity of plasma IgM and IgG from malaria-experienced children (n = 18) and adults (n = 24) against recombinant *P. falciparum* 3D7 P41, VFT, AMA1, Pf3D7_1136200, Pf113, and MSP3. **(E)** Reactivity of plasma IgM and IgG from malaria-experienced children (n = 18) and adults (n = 24) against whole merozoites by ELISA. Values shown are optical densities after subtraction of background values obtained using pooled unexposed plasma from US donors. Data shown in panels **(B–D)** were tested for statistical significance using a two-way repeated measures ANOVA, followed by comparisons between children and adults using Šídák's *post-hoc* test, corrected for multiple comparisons. ****P < 0.0001; **P < 0.01; *P < 0.05; ns, not significant.

we were unable to determine reactivity of mAbs isolated from IgM^+ MBCs against the three PfMSP1 variants from different *Pf* strains, because of the loss of antigen reactivity when these mAbs were expressed as IgG. Of note, most mAbs were derived from a single donor, limiting the conclusions we could draw from this experiment.

Plasma Antibody Responses Against PfMSP1 Are Dominated by IgG in Both Children and Adults

To expand on the results of cross-strain reactivity of mAb against PfMSP1 and to determine whether the $PfMSP1_{FL\text{-}3D7}$-specific MBC isotype differences we observed between children and adults are reflected in the plasma antibody response, we tested plasma samples from 24 adults and 18 children for IgM and IgG reactivity against full-length PfMSP1 from the three *Pf* strains (**Supplementary Table 1**). This experimental design provides a measurement of the overall antibody reactivity of the plasma but does not discriminate between antibodies with cross-strain reactivity or a combination of multiple strain-specific antibodies. However, it would reveal whether anti-PfMSP1 antibody responses broaden with cumulative *Pf* exposure. As a control, we included plasma samples from recovered U.S. COVID-19 patients (n = 10) that showed IgM and IgG reactivity against the SARS-CoV-2 receptor binding domain, but not against the three full-length PfMSP1 variants. In both children and adults, anti-PfMSP1 IgM reactivity in the plasma was very low, with the exception of three of the 24 adult samples that contained high IgM reactivity (MFI > 15×10^3) against at least one PfMSP1 variant antigen (**Figure 5B**). In contrast, all samples showed intermediate to high IgG reactivity against all PfMSP1 variants (**Figure 5C**). Adults had higher plasma IgG reactivity against $PfMSP1_{FL\text{-}HB3}$ than children. The average reactivity of adult samples against $PfMSP1_{FL\text{-}Dd2}$ was also higher but not statistically significantly different from that in children (P = 0.06). These results suggest that while children already have plasma IgG against different variants of PfMSP1, this response continues to broaden with age.

To determine whether the differential IgM and IgG reactivity of plasma is specific for anti-PfMSP1 antibodies or can be extrapolated to antibody responses against other merozoite antigens, we tested reactivity of plasma IgM and IgG to a panel of six recombinant merozoite antigens (all *Pf* 3D7 variants) by Luminex. We observed different reactivity patterns for these antigens (**Figure 5D**). Plasma reactivity to PfP41 and PfVFT was similar to $PfMSP1_{FL\text{-}3D7}$, with low IgM and high IgG reactivity in both children and adults. For PfAMA1, IgM reactivity was also low in children, but increased in adults. However, nearly all individuals showed IgM and IgG reactivity against Pf3D7_1136200, Pf113, and PfMSP3, which for Pf113 was higher in adults compared to children (**Figure 5D**). The ratio between IgG and IgM MFI values, where a high ratio is indicative of an IgG-dominant response, was higher for $PfMSP1_{FL\text{-}3D7}$ than for each of the six merozoite antigens in adults, and for Pf113, PfMSP3, and Pf3D7_1136200 in children (**Supplementary Figure 5**). Finally, we analyzed serum reactivity to whole merozoites from *Pf* strain 3D7. We observed IgM and IgG reactivity in both groups, with adults showing higher reactivity for both IgM and IgG as compared to children (**Figure 5E**). IgM and IgG anti-merozoite reactivity were not directly comparable since the values measured are dependent on the binding affinity of the secondary antibody. Collectively, these results suggest that in contrast to our observation that the PfMSP1-specific MBC response in children is enriched for IgM^+ MBCs, the plasma antibody response against PfMSP1 as well as to several other *Pf* merozoite antigens in both children and adults is dominated by IgG. However, based on our experiments with whole merozoites, IgM responses against other merozoite antigens appear better developed, particularly in adults.

The Anti-PfMSP1 Plasma IgG Repertoire Has Limited Diversity, High Levels of Amino Acid Substitutions, and Mainly Overlaps With Sequences Found in Classical Memory B Cells

To further explore the connection between the plasma cell and MBC compartments of the humoral immune response, we performed an integrative analysis of the plasma anti-PfMSP1 IgG and B cell receptor repertoires in adult donor 2, who was selected based on the availability of additional PBMCs and plasma. Although the results of this experiment will require confirmation in additional individuals, this is the first analysis of its kind in a malaria-experienced person and will provide valuable insight into the molecular characteristics of the anti-PfMSP1 plasma antibody repertoire after life-long exposure to *Pf*. In a previous study, we generated B cell receptor sequencing (BCR-seq) data of antibody heavy chain variable regions of naïve B cells, classical MBCs, and atypical MBCs (43). The full BCR-seq data set and all sequences obtained from PfMSP1-specific MBCs were used to construct a personalized, donor-specific reference heavy chain antibody variable region sequence database. We then isolated anti-PfMSP1 IgG from plasma using commercially available 19 kDa C-terminal fragment of *Pf* strain 3D7 PfMSP1 ($PfMSP1_{19\text{-}3D7}$), which limited our analysis to the most conserved domain of PfMSP1 (44, 45). We analyzed the anti-$PfMSP1_{19\text{-}3D7}$ IgG preparation by high-resolution liquid chromatography with tandem mass spectrometry and searched the obtained spectra against the donor-specific antibody variable region database (**Figure 6A**). This step allowed us to identify the full-length antibody sequences that these short peptide spectra were derived from. Eighteen anti-$PfMSP1_{19\text{-}3D7}$ IgG lineages were identified, of which four lineages made up over 75% of all plasma anti-$MSP1_{19\text{-}3D7}$ IgG (**Figure 6B** and **Supplementary Table 2**), suggesting that the anti-$PfMSP1_{19\text{-}3D7}$ plasma IgG repertoire may be relatively limited in diversity. The 18 antibody lineages were dominated by sequences that were found among IgG_1^+ and IgG_3^+ classical MBCs, all of which had

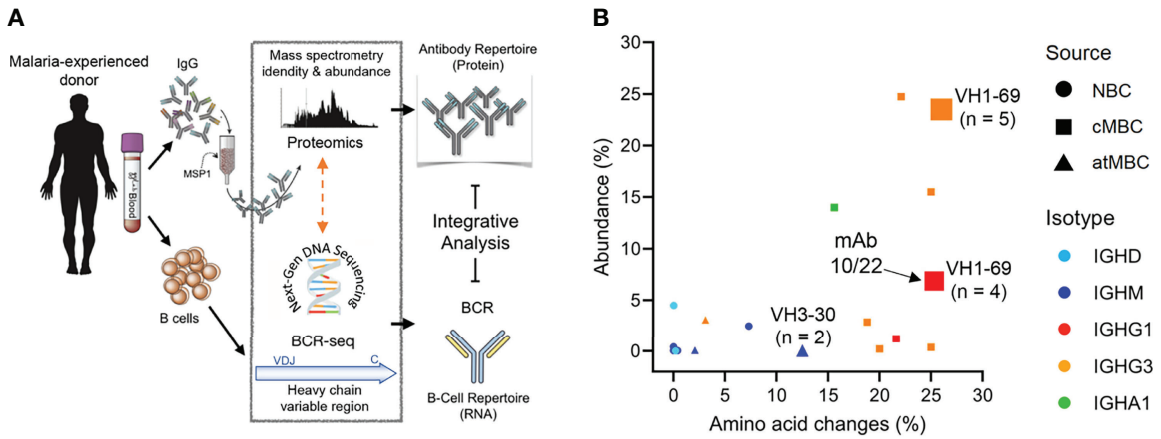

FIGURE 6 | Analysis of the repertoire of anti-PfMSP1$_{19\text{-}3D7}$ IgG in the plasma of a malaria-experienced adult. **(A)** Schematic overview of the experimental pipeline. **(B)** The percentage amino acid changes in the V gene segment of the heavy chain variable region (as determined by B cell receptor sequencing; X axis) plotted against the abundance of the corresponding IgG in plasma (Y axis). The size of the data points indicates the number of clonal sequences found in the B cell receptor sequencing (BCR-seq) data set, as a measure of the size of the clonal B cell lineage. The three lineages that were expanded in the BCR-seq data set are indicated with a number, that represents the number of clonal sequences identified by BCR-seq. The shape of the data point shows the B cell subset among which the sequence was found (NBC, naïve B cell; cMBC, classical memory B cell; atMBC, atypical memory B cell), while the color indicates the B cell isotype. The data point corresponding to mAb10 and mAb22 (isolated from PfMSP1-specific B cells) is indicated with an arrow.

relatively high levels of amino acid substitutions (>15%) (**Figure 6B**). These results suggest that PfMSP1-specific B cell lineages can give rise to both plasma cells and classical memory B cells, but not atypical MBCs. Two relatively abundant antibody lineages (comprising 23.5% and 6.9% of anti-PfMSP1$_{19}$ plasma IgG) overlapped with IgG$^+$ classical MBC lineages that were expanded in the bulk BCR-seq data (5 and 4 clonal B cell members, respectively). Both of these lineages used IGHV1-69 and IGHJ6 and had long HCDR3 sequences of 24 and 21 amino acid residues, respectively, suggesting that these characteristics may have contributed to the selection and expansion of both MBC and plasma cell populations. Long HCDR3s were also observed among the other IgG clonal lineages (average of all lineages, 18 amino acid residues; **Supplementary Table 2**), although the most abundant IgG (representing 24.7% of all anti-PfMSP1$_{19\text{-}3D7}$ plasma IgG) had an HCDR3 of 10 amino acid residues. A third antibody lineage with low abundance (<0.1%) matched with an expanded B cell lineage, consisting of two clonal IgM$^+$ atypical MBC members that had relatively high levels of amino acid substitutions (>10%). Except for these three expanded lineages, all other clonotypes were found as a single sequence in the bulk BCR-seq data set. These results highlight that the abundance of antibody clonotypes in the plasma is not necessarily reflected by an expansion of the corresponding MBC lineages, pointing towards different selective mechanisms that govern MBC and plasma cell development. It is important to point out that these results may have been influenced by the timing of sampling, and it can be expected to find more overlap between the MBC and plasma antibody repertoires during or shortly after infection.

Analysis of an Expanded B Cell Lineage Detected in Both Plasma IgG and Memory B Cells

To further analyze antibody characteristics that may influence their selection and expansion among plasma cells and MBCs, we compared the anti-PfMSP1$_{19\text{-}3D7}$ IgG sequences detected in plasma by mass spectrometry with those derived from the B cell receptors of PfMSP1$_{FL\text{-}3D7}$-specific MBCs that were obtained from the same donor at the same time point. Only the largest PfMSP1$_{FL\text{-}3D7}$-specific mAb lineage was detected among the anti-PfMSP1$_{19\text{-}3D7}$ antibody lineages identified in plasma, potentially because other PfMSP1$_{FL\text{-}3D7}$-specific mAbs target epitopes in other parts of the PfMSP1 protein. Alternatively, it is possible that B cell lineages are not often shared between MBCs and plasma cells. Two mAb sequences of this clonal lineage, mAb10 and mAb22, were found in plasma at a percentage of 6.8% and 0.1% of all anti-PfMSP1 IgG detected, respectively, making it the fifth largest clonotype in the circulation. Despite having highly similar heavy chain variable regions, including eight shared amino acid substitutions and a long HCDR3 sequence (21 amino acid residues, **Supplementary Figure 6**), mAb10 and mAb22 had different light chains. Like all other members of the clonal lineage (total n = 9), mAb22 had a lambda light chain, while mAb10 contained a kappa light chain. mAb10 was more abundant in plasma (6.8%), but was the only member of the MBC lineage with a kappa light chain. The opposite was observed for the lambda light chain variants, found at 0.1% of all anti-PfMSP1 plasma IgG, but representing eight out of nine members of the PfMSP1$_{FL\text{-}3D7}$-specific MBC lineage. This difference in relative abundance of lineage members in different B cell compartments may be related to the preferential

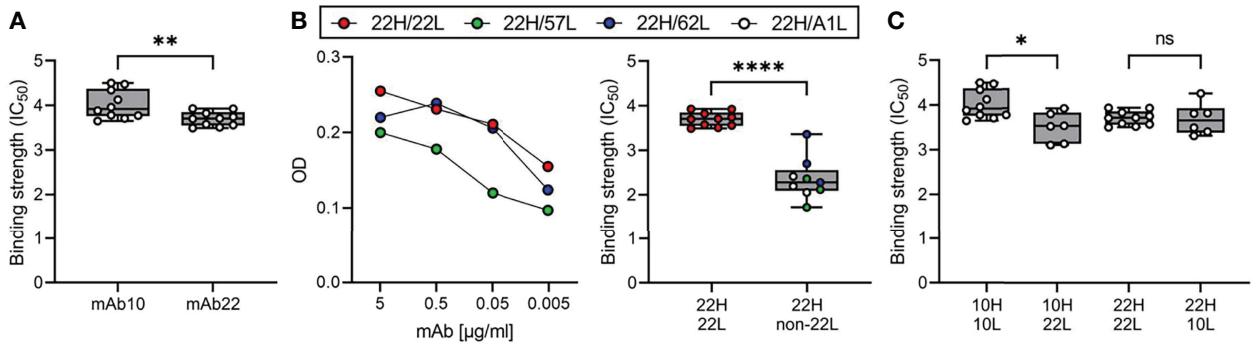

FIGURE 7 | Binding affinity of mAb10 and mAb22 to $PfMSP1_{3D7}$. **(A)** The binding strength of mAb10 and mAb22 as determined by chaotropic ELISA. Binding strength (IC_{50}) is defined as the concentration of urea that results in loss of 50% of mAb binding. A total of 10 replicates from three independent experiments are shown. Differences were tested for statistical significance using the Wilcoxon matched-pairs signed rank test, with pairs were defined as antibodies analyzed on the same plate. **(B)** The binding of mAb22 and antibodies that consisted of the heavy chain of mAb22 and a light chain of an unrelated, non-MSP1-reactive antibody to $PfMSP1_{3D7}$ by regular ELISA (left) and chaotropic ELISA (right). Differences in binding strength between replicates of mAb22 (n = 10) and chimeric antibodies (n = 9, three replicates for each of three unrelated light chains that were combined for analysis) were tested for statistical significance using the Mann Whitney test. **(C)** The binding strength of mAb10 and mAb22 (n = 10 replicates each) expressed with their own light chains or chimeric mAbs in which the light chains were swapped (n = 6 replicates each). In all panels, data points are the average of three technical replicates. Differences between antibodies with the same heavy chain but a different light chain were tested for statistical significance using a Kruskal-Wallis test, followed by comparisons between select groups using Dunn's *post-hoc* test, corrected for multiple comparisons. ****P < 0.0001; **P < 0.01; *P < 0.05; ns, not significant.

differentiation of high-affinity B cells to plasma cells and lower-affinity B cells to MBCs (46). For mAb10 and mAb22, it would therefore be expected that mAb10 has higher antigen-binding affinity. We determined binding strength of mAb10 and mAb22 to $PfMSP1_{FL\text{-}3D7}$ using a chaotropic ELISA with urea and observed that mAb10 indeed showed higher binding strength to $PfMSP1_{FL\text{-}3D7}$ than mAb22 (**Figure 7A**). The observation that mAb10 and mAb22 had different light chains suggests that antigen binding by these mAbs is dominated by their heavy chain. To test this, we expressed mAb22 with light chains from unrelated non-$PfMSP1_{FL\text{-}3D7}$-binding antibodies and observed that it was still reactive with $PfMSP1_{FL\text{-}3D7}$, albeit with lower binding strength (**Figure 7B**). These results suggest that the heavy chain of mAb22 (and presumably mAb10) is sufficient for binding to $PfMSP1_{FL\text{-}3D7}$, but that its light chain is important for optimal binding to antigen. This raised the question whether the difference in binding strength between mAb10 and mAb22 is caused by the different light chains used by the two mAbs. For each mAb, we therefore compared binding strength between the antibody expressed with its own light chain and a chimeric antibody in which the light chain was swapped. Expression of mAb10 with the mAb22 light chain resulted in a reduction of binding strength, while the binding strength of mAb22 was unchanged when expressed with the mAb10 light chain (**Figure 7C**). These results suggest that the light chain of mAb10 may play a role in the increased binding strength of mAb10 over mAb22, but is dependent on the mAb10 heavy chain for this effect. Interestingly, despite higher binding strength, mAb10 showed reduced activity in a growth inhibition assay as compared to mAb22 (**Figure 3C**). Collectively, these results highlight characteristics of the individual members of an PfMSP1-specific clonal B cell lineage that may have influenced their selection and fate.

DISCUSSION

The prevalence and magnitude of antibody responses against *Pf* merozoite antigens have been extensively studied [reviewed in (47, 48)]. In addition, it is known that *Pf* exposure results in the development of long-lived MBCs against merozoite antigens that are maintained in the absence of re-infection (28, 49–51). However, much remains unknown about the molecular characteristics of anti-merozoite antibodies and the connections between the MBC and plasma cell compartments. This information will increase our understanding of how durable B cell immunity develops and how this may be harnessed for vaccine development. Here, we compared the phenotype and molecular characteristics of MBCs against the most abundant merozoite surface antigen, PfMSP1, between a small number of children and adults living in a region of high *Pf* transmission in Uganda. In addition, we analyzed plasma IgM and IgG responses against PfMSP1 and other merozoite antigens in both children and adults from the same region. Finally, we analyzed the overlap between the MBC and plasma antibody compartments using mass spectrometry and B cell receptor sequencing for one adult to better understand the processes that drive B cell selection and fate decisions.

Our results showed that children harbored a larger fraction of PfMSP1-specific IgM^+ MBCs than adults, in line with a recent report (16). In contrast, the plasma anti-PfMSP1 antibody response was dominated by IgG. We recognize that it is difficult to directly compare IgM and IgG measurements due to potential differences between secondary antibodies used in these assays. However, our control samples from recovered COVID-19 patients demonstrate that we were able to measure IgM reactivity, yet we detected plasma IgM reactivity against PfMSP1 in only a small percentage of adults. These observations

suggest that malaria-experienced children develop a strong IgG response against PfMSP1 that is not reflected in the MBC compartment. A similar observation was made by Dorfman et al., who reported a disconnect between IgG responses to PfAMA1 and the presence of PfAMA1-specific IgG^+ MBCs in children (52). One explanation for the relative lack of IgG^+ MBCs in children could be that the B cell response predominantly gives rise to short-lived IgG^+ plasmablasts, whereas germinal center reactions are limited. Since germinal center responses are essential for the development of long-lived plasma cells and MBCs, this would explain the relatively quick waning of anti-parasite plasma IgG and MBCs in children in the absence of exposure, as seen in children living in malaria-endemic regions with a distinct dry season (18, 19). It has been shown that *Pf* infections can lead to dysregulation of various components of the immune system that are essential for the formation of germinal centers, including dendritic cells and T follicular helper cells (53–56). In addition, the abundant development of plasmablasts during *Plasmodium* infection in rodents directly limited the generation of germinal centers as a result of nutrient depletion (57). Thus, IgM^+ MBCs would mainly develop outside of germinal centers, which would be consistent with the low levels of somatic hypermutation that we observed. An alternative explanation for our observation could be that anti-PfMSP1 IgG^+ MBCs are formed equally efficiently in both children and adults but undergo recall shortly after their formation in children as a result of repetitive *Pf* infections with high parasitemia, whereas the lower antigenic load of asymptomatic infections in adults would allow for a longer lifespan of IgG^+ MBCs. This theory would be in line with the increase in the percentage of merozoite-specific MBCs with age that is observed in individuals who live in malaria-endemic regions (18). In support of this theory is our observation that the few IgG^+ MBCs isolated from children already had relatively high levels of amino acid substitutions, suggestive of multiple rounds of affinity maturation in germinal centers. It is also supported by our observation of a lack of clonal connections between IgG^+ MBCs at two time points six months apart in two five-year old children living in a high transmission region in Uganda, while we did find clonally related sequences among IgM^+ MBCs between the same two time points (58). These data could suggest that the IgG^+ MBC compartment in children undergoes more rapid turnover than the IgM^+ MBC compartment, or that the IgM^+ MBC compartment has more self-renewing capacity (59). Of note, our findings may be specific for PfMSP1 and the small sample size in many of our experiments precludes definitive conclusions. More longitudinal studies in the same individuals will be needed to track the fate of parasite-specific IgG^+ MBCs after their formation.

We observed a low level of plasma IgM against PfMSP1 as compared to plasma IgG. Although IgM reactivity was higher against other *Pf* antigens, the question of how much IgM contributes to the control of *Pf* infections remains unanswered. IgM responses against a variety of *Pf* antigens have been reported in several studies, in particular in high transmission regions (60–63), but there is conflicting evidence on the durability of the IgM response and its role in parasite inhibition. Boyle et al. reported that IgM responses against PfMSP2 were sustained in children living in Kenya during a period with low parasite transmission (14). In contrast, Walker et al. concluded that IgM responses against five other merozoite antigens decreased rapidly following *Pf* malaria in children living in Ghana, whereas IgG responses against these five antigens were maintained for a longer period of time (63). These conflicting observations may be the result of differences between the assays used to assess these antibody responses, or differences in the development of IgM responses under different transmission intensities or against different parasite antigens, as also observed in this study. An association between IgM reactivity against *Pf* antigens, including $PfMSP1_{19}$, PfAMA1, PfMSP3, and PfGLURP, and protection against malaria was reported by some (14, 64, 65), but not by others (66). When tested at the same concentration, purified plasma IgM from malaria-experienced individuals had equal opsonic phagocytosis activity and two-fold lower fixation capacity of C1q, the primary component of the classical complement pathway, as compared to plasma IgG, but induced nine-fold higher deposition of components of the membrane attack complex (14, 16). While these results suggest that IgM can indeed contribute to parasite inhibition, the concentration of total IgM in plasma is almost one order of magnitude lower than that of IgG [average, 1.5 g/l for IgM versus 11 g/l for IgG (67, 68)]. Therefore, the relative contributions of IgM and IgG to parasite inhibition remain to be determined. Finally, a passive immunization study showed that IgG-depleted plasma from malaria-experienced adults had no effect on parasitemia in children with *Pf* malaria, whereas treatment with IgG from the same individuals resulted in a dramatic decrease of parasite counts (69), suggesting that IgG is the main effector antibody responsible for parasite control.

The acquisition of high levels of amino acid substitutions in IgG^+ MBCs from both children and adults suggests that these cells are the product of multiple rounds of affinity selection. We also detected several B cells with long HCDR3s, similar to broadly neutralizing antibodies against HIV (70), although this was not a universal characteristic of anti-MSP1 mAbs. In addition, we observed that children and adults had equally high IgG reactivity against $PfMSP1_{FL\text{-}3D7}$, but that reactivity against $PfMSP1_{FL\text{-}HB3}$ was lower in children. This suggests that the IgG response continues to broaden with subsequent *Pf* infections, resulting in a larger fraction of IgG directed against conserved epitopes. The broadening of antibody responses, high mutational load, and expansion of clonal PfMSP1-specific B cell lineages is suggestive of strong selection pressure on the B cell response as a result of *Pf* infections.

We believe that our observations regarding mAb10 and mAb22, two members of an PfMSP1-specific IgG^+ MBC lineage that share the same heavy chain but have different light chains, are additional evidence of strong forces on B cell selection and development. The difference in light chain usage between mAb10 and mAb22 raises the question how these mAbs are developmentally related. One possibility is that mAb10 and

mAb22 were derived from a B cell precursor that underwent cell division upon heavy chain rearrangement in the bone marrow, giving rise to multiple daughter cells that each underwent light chain rearrangement independently. In this scenario, the shared amino acid mutations would be the result of convergent evolution selecting for higher affinity antigen binding. In a second scenario, a precursor B cell activated by antigen could have undergone secondary light chain rearrangement in the germinal center. In this case, the kappa light chain-bearing mAb10 would likely be evolutionarily closer to this precursor, and mAb22 (and all other MBCs in this lineage) would be derived from this secondary rearrangement event and have undergone further diversifying selection. We were unable to determine whether mAb10 and mAb22 developed as a result of convergent evolution or clonal lineage diversification. However, our results suggest that the light chains of these antibodies may affect antibody binding affinity. This could, on its own, have resulted in the preferential differentiation of the higher affinity mAb10 variant into a plasma cell and the lower affinity mAb22 variant into an MBC. Further studies into the relationship between the plasma cell and MBC compartments for PfMSP1 and other merozoite antigens are currently ongoing in our laboratories.

Given its higher binding affinity, the relatively low inhibition of parasite growth by mAb10 as compared to mAb22 is unexpected. One potential explanation is that inhibition as measured in the growth inhibition assay can be the result of multiple mechanisms, such as neutralization or prevention of proteolytic cleavage of PfMSP1. High binding affinity is likely to be important for optimal neutralization. For inhibition of proteolytic cleavage, on the other hand, the precise antibody epitope and orientation may be more important determinants. The kappa and lambda light chains of mAb10 and mAb22 may have different footprints on the surface of PfMSP1, thereby differentially occluding the cleavage site that releases $PfMSP1_{19}$, resulting in differences in growth inhibition. It will be interesting to determine the ability of these antibodies to induce opsonic phagocytosis or fix complement, as these measurements of parasite inhibition may be influenced more strongly by antibody affinity.

This study has several limitations. First, we analyzed PfMSP1-specific B cell phenotypes in a small number of individuals. However, the observed shift from predominantly IgM^+ PfMSP1-specific MBCs in children to IgG^+ PfMSP1-specific MBCs in adults was in agreement with results reported recently by Hopp et al., which provides strength to our observations. Second, the sequence analysis of mAbs cloned from PfMSP1-specific B cells and anti-MSP1 plasma IgG were performed using a small number of donors and small numbers of antibody sequences. We used stringent criteria for the inclusion of mAbs in downstream analyses and only analyzed the sequences of mAbs with confirmed PfMSP1 reactivity, which may have affected our observations with regard to antibody diversity and clonal expansion. Finally, we selected PfMSP1 as a model antigen for this study. An analysis of plasma antibody responses against other merozoite antigens highlighted differences in the plasma antibody response between PfMSP1 and PfMSP3, Pf113, and Pf3D7_1136200 (**Figure 5D**), suggesting that the results reported here apply to PfMSP1, but may not necessarily be representative of B cell responses to other *Pf* antigens.

In conclusion, we performed an analysis of the MBC compartment and antibody responses against PfMSP1 in children and adults living under high *Pf* transmission conditions. We observed that children in our study predominantly harbor PfMSP1-specific IgM^+ MBCs, while the MBC response shifted to IgG^+ B cells in adults. In contrast to this difference in isotype among PfMSP1-specific MBCs, both children and adults demonstrated strong anti-MSP1 plasma IgG responses, while anti-PfMSP1 plasma IgM responses were minimal. IgG^+ MBCs carried high levels of somatic hypermutations and clonal expansion, suggestive of ongoing B cell selection over the course of sequential *Pf* infections. Finally, we directly compared the overlap between the PfMSP1-specific MBC compartment and anti-PfMSP1 plasma IgG, revealing that the same molecular characteristics observed in the MBC compartment were also dominant in the plasma IgG compartment. Collectively, our results provide new insights into the development of B cell responses against *Pf*, in particular about the similarities and differences between MBC and antibody responses.

MATERIALS AND METHODS

Ethics Approval Statement

Participants were enrolled in the Tororo Child Cohort or the Program for Resistance, Immunology, Surveillance, and Modeling of Malaria (PRISM) Cohort, and provided written consent for the use of their samples for research. These cohort studies were approved by the Makerere University School of Medicine Research and Ethics Committee (SOMREC) and the University of California, San Francisco Human Research Protection Program & Institutional Review Board. The use of cohort samples was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. Donors 1 and 2 were anonymous blood donors at Mbale regional blood bank in Eastern Uganda, who consented to the use of their blood for research. The use of samples from anonymous blood donors was not considered human research by the Institutional Review Board of the University of Texas Health Science Center at San Antonio due to the lack of any identifiable information and was therefore exempt from review.

COVID-19 samples used in this study were received de-identified from the University of Texas Health San Antonio COVID-19 Repository. This repository was reviewed and approved by the University of Texas Health Science Center at San Antonio Institutional Review Board. All study participants provided written informed consent prior to specimen collection for the repository to include collection of associated clinical information and use of left-over clinical specimens for research. The COVID-19 Repository utilizes an honest broker system to maintain participant confidentiality and release of de-identified data or specimens to recipient investigators.

Participants

Malaria-experienced participants (n = 46) were residents of Eastern Uganda, a region with extremely high malaria transmission intensity (annual entomological inoculation rate in the region estimated at 125 infectious bites per person per year (71). In this region, children between five and ten years of age start to develop protective immune responses against malaria, as evidenced by a record of parasitemia in the absence of malaria, but are typically still susceptible to disease. Children above ten years of age and adults have developed protective immune responses, evidenced by no signs of clinical malaria despite high exposure, demonstrated by household entomological and epidemiological measures, including documented asymptomatic parasitemia. In addition to selection based on age, sex was used as an inclusion criterium to ensure equal representation of males and females among the children, and to include as many males as possible for the adults. The PRISM cohort provided health facility-based malaria surveillance. All children 6 months to 10 years old within a household and up to one primary adult caregiver (often female) were enrolled in a dynamic cohort, and children were excluded from follow-up after reaching 11 years of age. Participants had routine clinic visits roughly every 3 months and attended a study clinic any time they became ill.

COVID-19 patients (n = 10) were enrolled in the University of Texas Health San Antonio COVID-19 Repository. Blood was collected after discharge from the hospital, on average approximately 30 days after symptom onset. These donors were included as malaria-naïve controls.

MSP1 Expression and Tetramer Synthesis

To produce C-terminal biotinylated full-length 3D7 PfMSP1 ($MSP1_{FL\text{-}3D7}$), human Expi293F cells (Thermo #A14635) were cultured, passaged, and transfected with the plasmids MSP1-bio (Addgene #47709) and secretedBirA-8his (Addgene #32408) at a 4:1 (w/w) ratio according to Thermo's protocol. Both plasmids were a kind gift from Gavin Wright (72, 73). Biotin (Thermo #PI21336) was added to a final concentration of 100 µM immediately after adding the transfection mix. Cell were cultured in either non-baffled polycarbonate flasks with a vent cap (Fisher #PBV12-5) or in glass Erlenmeyer flasks loosely covered with aluminum foil to allow for gas exchange. Cells were successfully passaged in volumes as low as 5 ml. Absence of mycoplasma contamination was confirmed using the MycoAlerta Plus mycoplasma detection kit (Lonza #LT07705). Culture supernatants were collected 5 – 7 days post-transfection by centrifuging the culture at 4,000 × g for 25 min. at RT. A 10 kDa cutoff Protein Concentrator PES (Thermo #88527) was used (5,000 × g at 4°C) to exchange culture medium containing free biotin for PBS (pH 7.2) (> 100,000 dilution) and to concentrate the protein to a final volume of 0.5 – 1 ml. The 3D7 PfMSP1 protein was mixed with 6 – 12 volumes of PBS (pH 5.5) in a final volume of six ml and was subsequently loaded onto gravity flow columns (Thermo #29924) containing CaptAvidin agarose (Thermo #C21386) for purification. After three washes with PBS (pH 5.5) and five 6 ml elutions with PBS (pH 10.5), the elutions were pooled (30 ml) and the pH was immediately neutralized by adding 12 ml PBS (pH 5.5). After concentrating, the protein was quantified using the Coomassie Plus (Bradford) Assay Kit (Thermo #23236) on a NanoDrop One spectrophotometer, according to the manufacturer's instructions, visualized by SDS-PAGE (see methods below; **Supplementary Figure 7A**), diluted to 1 mg/ml, aliquoted, and stored at -70°C.

Since each streptavidin molecule has the ability to bind four biotinylated $PfMSP1_{FL\text{-}3D7}$ molecules, $PfMSP1_{FL\text{-}3D7}$ tetramers were made by incubating $PfMSP1_{FL\text{-}3D7}$ in a tube revolver (Thermo #88881001) at 40 rpm and RT for 30 min. with streptavidin-PE (Thermo #S866) at a 6:1 molar ratio. After this incubation, the tetramers were washed with PBS (pH 7.2) using a Vivaspin centrifugal concentrator (Sartorius #VS0141) three times for 5 min. at 15,000 × g at RT. To make decoy tetramers, streptavidin-PE was first conjugated to Alexa-fluor 647 (Thermo #A20186) per manufacturer's instructions. This double-conjugated streptavidin was then coupled to *R. norvegicus* CD200 [Addgene #36152 (73)] as described above.

SDS-PAGE

As a quality control step, purified proteins were visualized on a polyacrylamide gel. Purified samples were mixed with Laemmli buffer and NuPage sample reducing agent (Thermo #NP0004; not added for monoclonal antibodies), incubated at 85°C for two min. The samples were run on a 4 – 12% Bis-Tris gel (Thermo #NP0321BOX) with MOPS running buffer (Thermo #NP0001) at 200 V for 50 min. The proteins were stained using Imperial Coomassie protein stain (Thermo #PI24615) per manufacturer's instructions.

MSP1-Specific B Cells Isolations

Peripheral blood mononuclear cells (PBMCs) from malaria-experienced individuals were cryopreserved in liquid nitrogen at -196°C and continuously kept at this temperature during storage and transport to minimize changes to B cell phenotypes (74). Cryopreserved PBMCs from malaria-experienced children and adults were thawed in a water bath at 37°C and immediately mixed with pre-warmed thawing medium [IMDM Glutamax (Thermo #31980030) supplemented with 10% heat-inactivated fetal bovine serum (FBS) of US origin (Sigma #TMS-013-B) and 33 U/ml universal nuclease (Thermo #88700)] and then centrifuged (5 min. at 250 × g and RT). The cell pellet was resuspended in thawing medium and viable cells were counted by adding 10 µl filtered 0.2% trypan blue in PBS to 10 µl of the cell suspension on a Cellometer Mini (Nexcelom) automated cell counter. Next, cells were pelleted by centrifugation (5 min. at 250 × g and RT) and resuspended in isolation buffer (PBS supplemented with 2% heat-inactivated FBS and 1 mM EDTA) at 50 million live cells/ml and filtered through a 35 µm sterile filter cap (Corning #352235) to break apart any aggregated cells. B cells were isolated using StemCell's EasySep Human B Cell Isolation Kit (#17954) according to manufacturer's instruction. After washing with PBS, the isolated B cells were incubated with 1 µl LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo #L34965) per 1 ml cell suspension, per manufacturer's instructions. After washing the B

cells with cold PBS and resuspending them in 50 µl cold PBS with 1% bovine serum albumin (BSA) (Sigma #A7979), the cells were first stained with 40 nM of decoy tetramer (10 min. in the dark on ice) and then with 20 nM of $PfMSP1_{FL\text{-}3D7}$ tetramer (30 min. in the dark on ice), followed by a wash with 1 ml of cold PBS/1% BSA (5 min. at 250 × g and RT). Tetramer-bound B cells were selected using StemCell's EasySep Human PE Positive Selection Kit (#17664) and subsequently stained on ice for 30 min. with an antibody panel against B cell surface markers (**Supplementary Table 3**). UltraComp eBeads (Thermo #01222242) were used to prepare compensation controls for each fluorophore per manufacturer's instructions. Before acquisition on a BD FACSAria II cell sorter, the cells were washed with 3 ml of cold PBS with 1% BSA (5 min. at 250 × g and 4°C), diluted to 20 – 30 million cells/ml in PBS with 1% BSA, and filtered into a FACS tube with filter cap. Lymphocytes were gated using forward and sideward scatter, followed by doublet exclusion and gating on live cells. PfMSP1-specific mature IgG^+ and IgM^+ B cells ($CD19^+$, $CD20^+$) were gated (PE^+, $AF647^-$) and single cells were sorted into 100 µl IMDM/Glutamax/10% FBS in a well of a 96-well plate (Corning #353072). One day prior to the sort, each well was seeded with 30,000 adherent, CD40L-expressing 3T3 cells (kind gift from Dr. Mark Connors, NIH) in 100 µl IMDM/Glutamax/10% FBS containing 2× MycoZap Plus-PR (Lonza #VZA-2021), 100 ng/ml human IL-2 (GoldBio #1110-02-50), 100 ng/ml human IL-21 (GoldBio #1110-21-10), 5 µg/ml TLR9-activator ODN2006 (IDT DNA, sequence TCGTCGTTTTGTCGTTTTGTCGTT), and 60 µg/ml transferrin (Sigma #616424) to promote expansion and differentiation of B cells into antibody-secreting cells (35, 36). After incubation at 37°C and 8% CO_2 for two weeks, the wells were screened for the production of IgM or IgG by enzyme-linked immunosorbent assay (ELISA).

Parasite Culture and Merozoite Isolation

Pf strain 3D7 parasites were cultured (75) in human AB^+ erythrocytes (Interstate Blood Bank, Memphis, TN, USA) at 3 – 10% parasitemia in complete culture medium (5% hematocrit). Complete culture medium consisted of RPMI 1640 medium (Gibco #32404014) supplemented with gentamicin (45 µg/ml final concentration; Gibco #15710064), HEPES (40 mM; Fisher #BP3101), $NaHCO_3$ (1.9 mg/ml; Sigma #SX03201), NaOH (2.7 mM; Fisher #SS266-1), hypoxanthine (17 µg/ml; Alfa Aesar #A11481-06), L-glutamine (2.1 mM; Corning #25005Cl), D-glucose (2.1 mg/ml; Fisher #D16-1), and 10% heat-inactivated human AB^+ serum (Valley Biomedical #HP1022). Parasites were cultured at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Before use in cultures, 12.5 ml packed erythrocytes were washed twice with 10 ml cold incomplete medium (complete culture medium without human serum) and pelleted between each wash by centrifugation at 500 × g for 8 min. at 4°C (max. acceleration and weakest break). Washed erythrocytes were resuspended in 2 volumes of complete medium and stored at 4°C.

Parasites were synchronized to the ring stage by treatment with 5% D-sorbitol (76) (Fisher #S459-500). Cultures containing high percentages of ring-stage parasites were centrifuged at 250 × g for 5 min. at RT. Pelleted erythrocytes were resuspended in 10 volumes of 5% D-sorbitol in MQ water, vortexed for 30 sec. and incubated for 8 min. at 37°C. The cells were then washed with 5 volumes of complete culture medium (250 × *g* for 5 min. at RT) and resuspended in complete culture medium at 5% hematocrit and cultured as described above. To obtain tightly synchronized parasites, sorbitol treatments were performed twice, 14 hours apart.

Infected erythrocytes containing parasites in the late-trophozoite and schizont stages were isolated from culture by magnetic separation (77, 78). Late-stage parasites were separated from uninfected and ring-infected erythrocytes with a SuperMACS II Separator (Miltenyi #130-044-104). The magnet was assembled with a D column (Miltenyi #130-041-201) according to manufacturer's instructions. The column was equilibrated with 200 ml incomplete medium. An additional 50 ml incomplete medium was added to the column through the side syringe to remove air bubbles possibly remaining in the column matrix. A 22 G needle (BD #305155) was attached to the stopcock to serve as a flow restrictor. For safety purposes the plastic protective sheath remained on the needle after cutting the end to allow flow of the liquid without exposing the tip of the needle. Approximately 100 – 200 ml of synchronized parasite culture (5 – 10% parasitemia, 5% hematocrit) 24 – 27 hours following the second sorbitol treatment (majority of parasites in the early segmented schizont stage, 4 – 6 nuclei visible by Giemsa staining) were used for merozoite isolation. After passing the parasite culture through the column, the column was washed from the top with incomplete medium until the flowthrough was clear (usuall ~100 ml). Next, the column was washed with a total of 150 ml incomplete medium (50 ml from the side and 100 ml from the top). Erythrocytes containing late-stage parasites with high paramagnetic hemozoin levels are preferentially retained in the column matrix while attached to the magnet (79) allowing for separation of late-stage parasites from uninfected erythrocytes and early-stage parasites. The column was removed from the magnet and 60 ml incomplete medium was used to elute the erythrocytes from the column matrix. The erythrocytes were pelleted by centrifugation at 250 × *g* for 5 min. at RT and were resuspended in 3 ml complete culture medium. Infected erythrocytes were incubated with E64 (10 µM final concentration, Sigma #324890-1MG) for 8 hours at normal culture conditions to allow the parasites to develop into fully segmented schizonts while preventing egress from the erythrocytes. Infected erythrocytes containing schizonts were then pelleted by centrifugation at 1,900 × *g* for 8 min. at RT and the supernatant containing E64 was removed. A thin smear from the pellet was Giemsa stained and merozoite yield was assessed by counting the number of fully segmented schizonts present. The pellet was resuspended in 4 ml incomplete medium. Merozoites were released from the erythrocytes by passing them through a 1.2 µm syringe filter (Pall #4190) and were subsequently pelleted by centrifugation at 4,000 × *g* for 10 min. at RT. On average, 5×10^7 merozoites were collected per 25 ml of synchronized culture. The merozoites were resuspended in PBS and stored at 4°C for up to one day until used for the ELISA.

Enzyme-Linked Immunosorbent Assays

To detect IgG and IgM, 96-well ELISA plates (Corning #3361) were coated with either goat anti-human IgG (Sigma #I2136) or IgM (Sigma #I1636) antibody at a concentration of 4 and 8 μg/ml, respectively, diluted in PBS, at a total volume of 100 μl per well. After a one hour incubation at 37°C or O/N at 4°C, each well was washed once using slowly running (approximately 900 ml/min.) deionized water. This washing method resulted in significantly higher specificity than other methods tested in the lab (using a plate washer with water or PBS containing 0.1% tween-20, or a squeeze bottle filled with PBS containing 0.1% tween-20). All subsequent washes were performed this way. 150 μl blocking buffer (one-third Non-Animal Protein (NAP)-Blocker (G-Biosciences #786-190P) and two-thirds PBS) was added to each well to prevent non-specific binding. After one hour of incubation at 37°C, the wells were washed three times and 50 μl B cell culture supernatant diluted 1:1 in dilution buffer (1% NAP Blocker in PBS; total volume 100 μl) was added per well. Plates were incubated for two hours at 37°C and washed five times. Then, either 100 μl 1:2500 diluted (1% NAP Blocker in PBS) HRP-conjugated anti-human IgG antibody (BioLegend #410902) or 1:5000 HRP-conjugated anti-human IgM antibody (Sigma #AP114P) was added to each well. After incubation for one hour at 37°C and three washes, HRP activity was detected using 50 μl TMB (Thermo #PI34024). Plates were incubated in the dark at RT and the oxidation reaction was stopped by adding 50 μl 0.18M H_2SO_4 (Fisher #FLA300-212) per well when the negative controls (wells that received buffer when test wells received culture supernatant) started to color. Absorbance was measured at 450 nm using a BioTek Synergy H4 microplate reader. A human IgG (Sigma #I2511) or IgM (Sigma #I8260-1MG) standard curve (ten three-fold serial dilutions starting at 20 μg/ml) was used to quantify samples. Wells with values >27 ng/ml were considered positive. This cutoff was determined based on our observation that the amplification of heavy and light chain variable regions always failed from cultures with a lower concentration.

ELISAs to confirm reactivity of $PfMSP1_{FL\text{-}3D7}$-specific antibodies were performed as described above with the following modifications. Plates were coated with 50 μl in-house produced $PfMSP1_{FL\text{-}3D7}$ per well at a concentration of 16 μg/ml (0.8 μg/well). Coated plates were incubated for 1 hour at 37°C or overnight at 4°C and all subsequent incubations were done at RT instead of 37°C. To prevent non-specific binding, the wells were blocked with 200 μl PBS containing 0.1% tween-20 and 3% non-fat milk powder (SACO), which significantly increased specificity of the assay (compared to NAP blocker). After discarding the blocking buffer from the wells, the plates were not washed. Purified antibodies were tested at a final concentration of 2.5 μg/ml in 100 – 200 μl in PBS containing 0.1% tween-20 and 1% non-fat milk powder. The plates were washed six times prior to adding the detection antibody, and four times prior to adding TMB substrate. To analyze binding strength of monoclonal antibodies to $PfMSP1_{FL\text{-}3D7}$, chaotropic ELISAs were performed similar to the above descriptions, with the following modifications. Wells were coated with 0.2 μg protein and the final concentration of the antibodies that were tested was 0.5 μg/ml. Following the incubation with anti-MSP1 antibodies, the plates were washed four times. Then, urea (Fisher #U15-500) was added to wells at the following concentrations: 0, 1, 2, 3, 4, 5 and 8M (in 100 μl PBS with 0.1% tween-20 and 1% milk powder). After a 15-minute incubation at RT, plates were washed four times. The IC_{50} (the molar concentration of urea required to reduce antibody binding to PfMSP1 by 50%) was calculated using non-linear regression analysis in GraphPad Prism 9. Urea concentrations were log-transformed prior to analysis. OD values for each technical replicate were normalized by setting the smallest OD to 0% and the largest OD to 100%. The IC_{50} values of three technical replicates were averaged to obtain the final IC_{50} for an experiment.

ELISAs to detect antibody reactivity against merozoites were performed as described for PfMSP1-specific antibodies above with the following modifications. Free merozoites were coated at 500,000 merozoites per well in 100 μl PBS, followed by overnight incubation at 4°C. Merozoite count was estimated based on culture parasitemia. Plasma samples were tested at a 1:200 dilution in a total volume of 100 μl.

Luminex Assay

Recombinant proteins were produced in Expi293F cells as described above, quantified using the Coomassie Plus (Bradford) Assay Kit (Thermo #23236) on a NanoDrop One spectrophotometer, according to the manufacturer's instructions, and visualized on SDS-PAGE gel to confirm protein size and purity (**Supplementary Figures 7A, B**). 100 pmol $PfMSP1_{FL\text{-}3D7}$, $PfMSP1_{FL\text{-}Dd2}$, $PfMSP1_{FL\text{-}HB3}$, PfMSP3, PfP41, Pf113, Pf3D7_1136200, PfVFT, PfAMA1, and SARS-CoV-2 receptor binding domain were coupled per 1×10^6 MagPlex microspheres (Luminex, #MC10025-ID) using the Luminex protein coupling kit (#40-50016) per manufacturer's instructions. All subsequent steps were done at RT and the beads were protected from light using aluminum foil. Coupled beads were pooled, resuspended in buffer A (PBS with 0.05% Tween 20 (Fisher #BP337), 0.5% BSA (Sigma #A7979), 0.02% sodium azide) and plated at 1000 beads per well for each protein in a black, flat-bottom 96 well plate (Bio-Rad #171025001). The beads were washed once. All washes were done with 100 μl PBST (PBS with 0.05% Tween 20) using a handheld magnetic washer (Bio-Rad #171020100). The incubation time on the magnet was always 2 min. Next, the beads were incubated with 50 μl purified anti-MSP1 antibody (diluted to 1 μg/ml using buffer B) or B cell culture supernatant (diluted 1:1 with buffer B) for 30 min. with constant agitation (500 rpm, 2.5 mm orbital diameter). Buffer B (0.05% Tween-20, 0.5% BSA, 0.02% sodium azide, 0.1% casein (Sigma #C7078), 0.5% PVA (Sigma #P8136) and 0.5% PVP (Sigma #PVP360), 15 μg/ml *E. coli* lysate) was prepared a day prior to the assay since it required the chemicals to dissolve O/N. On the day of the assay, *E. coli* lysate (MCLAB #ECCL-100) was resuspended in MQ water and added to a final concentration of 15 μg/ml. Prior to use, buffer B was centrifuged at 10,000 × g for 10 min. After three washes, 50 μl secondary antibody diluted in buffer A (PE anti-human IgG (1:200 dilution;

Jackson ImmunoResearch #109-116-098)) or PE anti-human IgM (1:80 dilution; BioLegend #314507)) was added per well. After 30 min. incubation with constant agitation, the beads were washed three times and subsequently incubated in 50 µl buffer A for 30 min. with constant agitation. After one final wash, the beads were resuspended in 100 µl PBS and fluorescence intensity was measured using a calibrated and validated Bio-Rad Bio-Plex 200 machine.

Amplification of Antibody Heavy and Light Chain Variable Regions

$MSP1_{FL\text{-}3D7}$-specific B cells that successfully expanded in culture were collected by centrifugation (5 min. at 250 × g and RT) and stored at -70°C in 50 µl Tri-Reagent (Zymo #R2050-1-200). Heavy and light chain variable regions were amplified from PfMSP1-specific B cells by cDNA synthesis and a series of PCR reactions, shown in **Supplementary Figure 8A**. All primer sequences can be found in **Supplementary Table 4**. mRNA was isolated using Zymo's Direct-zol RNA Microprep kit (#R2060), eluted in 15 µl elution buffer and then mixed with 0.7 µl reverse primer (10 µM, 200 mM final concentration (f/c) in 35 µl PCR reaction volume) specific for the IgG, or IgM, heavy chain (primers #7 and #297) plus 0.7 µl light chain specific reverse primers (10 µM): #108 and #109), and incubated for two minutes at 65°C. Single stranded cDNA was synthesized immediately by adding 0.7 µl SMARTScribe reverse transcriptase (100 U/µl, f/c 2 U/µl, Takara Bio #639537), 7 µl First-Strand buffer (f/c 1×), 7 µl DTT (20 mM, f/c 4 mM), 0.7 µl dNTPs (10 mM each, f/c 200 µM each, Sigma #DNTP-10), 1.75 µl RNase OUT (40 U/µl, f/c 2 U/µl, Thermo #10777019), 0.7 µl template switch oligo (TSO) (10 µM, f/c 200 nM, IDT DNA; #110, **Supplementary Table 4**), nuclease-free water till 35 µl and subsequent incubation at 42°C for 2 hours. The TSO was designed with two isodeoxynucleotides at the 5' end to prevent TSO concatemerization and three riboguanosines at the 3' end for increased binding affinity to the appended deoxycytidines (property of the Takara reverse transcriptase) (80, 81). The single-stranded cDNA was immediately purified using Zymo's RNA Clean & Concentrator kit (#R1016) using Zymo's appended protocol to purify fragments >200 nucleotides and was eluted in 10 µl elution buffer. This critical clean-up step ensured that any unused TSO was removed, preventing it from inhibiting the subsequent PCR reactions by serving as template for the forward primer. Immediately after, heavy and light chain variable regions were amplified by PCR in one reaction mix using 8.5 µl purified cDNA, 10 µl AccuStart II PCR SuperMix (QuantaBio #95137), 0.9 µl 10 µM forward primer #106 (f/c 0.45 µM, **Supplementary Table 4**), and 0.2 µl of the reverse primers (10 µM) used to synthesize the cDNA (#7, #297, #108, and #109, each at f/c 0.1 µM). Cycling conditions were 94°C for 3 min., 35 cycles of 30 sec. at 94°C, 30 sec. at 55°C and 35 sec. at 72°C, followed by 5 min. at 72°C. A second, nested amplification was required to obtain enough amplicon DNA, and was done separately for heavy chain, kappa light chain, and lambda light chain variable regions, using AccuStart II PCR SuperMix, and 2 µl of the first, unpurified PCR as template in a total reaction volume of 20 µl. Mixes of primers (**Supplementary Figure 8A**) as described by Hua-Xin Liao et al. (82) were used for this second PCR, with a final concentration of 0.1 µM for each individual primer. Reverse primer #67 was added for the heavy chain variable region PCR to allow for amplification of variable regions originating from IgG_2, IgG_3 and IgG_4 mRNA, in addition to #30 which was specific for IgG_1. Cycling conditions were as described above, except for the extension step (shortened to 30 sec.) and the annealing step, which was 30 sec. at 60°C for the IgG1 heavy chain variable region, 30 sec. at 63°C for the IgM heavy chain variable region, and 30 sec. at 50°C for the light chain variable regions.

Linear IgG expression cassettes (82) were synthesized by PCR using 3 overlapping DNA fragments: a promoter (705 bp), a variable region and a constant region (IgG_1 heavy chain: 1188 bp; pentameric IgG_1 heavy chain: 1326 bp; kappa light chain: 568 bp; lambda light chain: 534 bp). Details about the assembly of linear expression cassettes are described by Liao et al. (82). All fragments were amplified in a 100 µl reaction using 20 ng plasmid template [HV0023 – HV0026 (82) or the IgG_1 expression plasmid containing the IgM multimerization sequence (see below)], 4 µl forward primer (10 µM), 4 µl reverse primer (10 µM), and QuantaBio AccuStart II PCR supermix (2×) using the following cycling program: 94°C for 3 min., 35 cycles of [94°C for 30 sec., 68°C for 30 sec., and 72°C for 40 – 75 sec. (1 min. per 1000 bp)] and 72°C for 5 min. Primers 53 and 54 were used for the promoter fragment, 55 and 58 for the heavy chain constant region fragment, 56 and 58 for the kappa light chain constant region fragment, 57 and 58 for the lambda light chain constant region fragment, and 55 and 494 for the pentameric IgG_1 heavy chain constant region fragment (**Supplementary Table 4**). The overlapping PCRs were done as follows. The cycling program was the same for all overlapping PCRs: 98°C for 1 min., 30× (98°C for 20 sec., 68°C for 15 sec., 72°C for 60 sec.), 72°C for 10 min. Two ng of each fragment (promoter, constant region, variable region) was used as template in a 25 µl PCR reaction with 2× KAPA HiFi Hot Start Ready Mix (Roche #KK2602) and 1 µl (10 µM) of the following forward and reverse primers: 50 and 51 for the IgG1 heavy chain, 50 and 52 for the kappa and lambda light chain, and 50 and 469 for the IgG_1 heavy chain with IgM multimerization domain (**Supplementary Table 4**). The final size of the linear expression cassettes was ~2300 bp for the IgG1 heavy chain, ~2400 bp for the IgG1 heavy chain with IgM multimerization domain, and 1600 bp for the kappa and lambda light chains (**Supplementary Figure 8B**). All linear expression cassettes were purified and sequence verified by Sanger sequencing. Variable region sequences were analyzed with IMGT/V-QUEST (38) using default settings to identify V(D)J gene usage and amino acid substitutions.

Generation of Antibody Expression Plasmids

Antibody variable regions were cloned into expression plasmids from *In vivo*gen (#pfusess-hchg1, #pfuse2ss-hclk, #pfuse2ss-hcll2). The variable heavy and light chain regions were amplified from the linear expression cassettes (2 µl at 1 ng/µl)

using 10 µl NEB Q5 Hot Start HiFi PCR master mix (#M0494S), 6 µl nuclease-free water and 1 µl sequence-specific F and R primer (10 µM, f/c 500 nM) that were based on the results of analysis using IMGT/VQUEST (38). These primers introduced restriction sites (EcoRI & NheI for hchg1, EcoRI & BsiWII for hclk, and EcoRI & AvrII for hcll2). Annealing temperatures were primer sequence dependent and were calculated using NEB's Tm calculator to match the salt concentration in their buffer. In an attempt to express the variable regions from IgM^+ B cells as a multimer (pentamer/hexamer mix) instead of a monomer, we modified the IgG_1 heavy chain expression plasmid (15). The IgM multimerization sequence PTLYNVSLVMSDTAGTCY (CCAACGCTCTATAATGTCTCTTTGGTTATGTCCGACA CAGCCGGTACCTGCTAT) was cloned into the IgG_1 expression vector at the C-terminus of the open reading frame, immediately in front of the stop codon, and the leucine at position -139 relative to the proline in the multimerization sequence was changed into a cysteine. Every plasmid was Sanger sequence-verified prior to using it as expression vector.

Antibody Expression and Purification

For small scale screening, one ml Expi293F cell cultures in a 6 well plate were transfected with heavy and light chain linear expression cassettes (1:1 molar ratio) according to the manufacturer's instructions for 25 – 30 ml cultures (also at 125 rpm).

Heavy and light chain antibody expression plasmids were used at a molar ratio of 1:2 to transfect 5 ml cultures. The antibodies were purified from the culture supernatant 4 – 6 days later using protein G magnetic beads (Promega #G7472). Purified antibodies and antibody elution buffer [5 parts elution buffer (100 µM glycine-HCl, pH 2.7) and 1 part neutralization buffer (2M Tris buffer, pH 7.5)] were buffer exchanged to PBS using 100 kDa cutoff Protein Concentrators (Thermo #88523). The samples were diluted > 50,000 × in PBS by repeated centrifugation at 4,000 × g and 4°C. Purified antibodies were quantified using the Coomassie Plus (Bradford) Assay Kit (Thermo #23236) on a NanoDrop One spectrophotometer, according to the manufacturer's instructions, and visualized on SDS-PAGE gel with a standard amount of BSA to confirm protein size and purity (**Supplementary Figure 7C**).

Immunofluorescence Assay

All steps of the immunofluorescence assay were done at RT. A thin blood smear was made on a microscopy slide from a 1 µl drop of E64-treated schizont culture. After drying for 30 sec., the cells were fixed by loading 1 ml of 4% paraformaldehyde (Electron Microscopy #15710) on the slide and incubating it for 30 min. The fixed cells were then washed three times with 1 ml PBS. Following the washes, the cells were permeabilized with 0.1% Triton-X (Fisher # BP151) in PBS and incubated for 30 min. The cells were then washed for an additional three washes using PBS. The slide was treated with blocking buffer (2% BSA, 0.05% Tween-20, 100 mM Glycine, 3 mM EDTA and 150 mM NaCl in PBS) for one hour. PfMSP1-specific mAbs were added to the cells at 1 µg/ml in 500 µl blocking buffer and incubated for one hour. Samples were then washed again three times using PBS. Goat anti-human IgG conjugated to FITC (Thermo #A18830) secondary antibody was diluted 1:1000 (1.5 µg/ml) in blocking buffer and then added to the smear to incubate for one hour in the dark. Samples were again washed three times using PBS in the dark and then allowed to air dry for one hour in the dark. Slides were mounted using 10 µl ProLong Glass mounting medium containing NucBlue Stain (Thermo #P36985) and sealed with a cover slip. Samples were imaged using a Zeiss Axio Imager Z1 with Zen Blue software.

Growth Inhibition Assay

Pf isolate 3D7 parasites were pre-synchronized at the ring stage with a 5% D-sorbitol (Fisher #S459-500) treatment as described above, followed four days later by two additional 5% D-sorbitol treatments 14 hours apart (76). At the late trophozoite/early schizont stage (24 hours after the third D-sorbitol treatment), parasitemia was determined by inspection of a Giemsa-stained blood smear. The smear was also used to confirm correct parasite staging. Immediately after, 20 µl of each antibody (1 mg/ml in PBS) was added to wells containing 30 µl complete medium in a black clear bottom 96-well plate (Corning #3603). A monoclonal antibody specific for apical membrane antigen 1 (AMA1) was used as a positive control (BEI #MRA-481A). Antibody elution buffer (100 mM glycine-HCl, pH 2.7) that was buffer exchanged to PBS alongside purified antibodies (see "Antibody expression and purification" above) was used as a negative control. Fifty µl parasite culture (1% parasitemia and 2% hematocrit) was then added to wells containing antibody or negative control. Uninfected erythrocytes (2% hematocrit) were used to determine the background signal. The plate was then incubated at standard parasite culture conditions (described above) for 48 hours before being transferred to a -70°C freezer. After overnight incubation of the plate at -70°C, SYBR green dye (Invitrogen #S7585) was added to lysis buffer (20 mM Tris-HCl (pH7.5), 5 mM EDTA, 0.008% saponin (Sigma # 558255100GM), 0.08% Triton X-100 in MQ water) at 0.2 µl dye per ml of lysis buffer. One hundred µl SYBR green lysis buffer was added to each well and the plate was incubated in the dark at 37°C for 3 – 6 hours. Fluorescence (excitation = 495 nm, emission = 525 nm, cutoff = 515 nm) was measured with a BioTek Synergy H4 plate reader. The instrument was programmed to read the plate from the bottom after mixing for 5 sec. The average background fluorescence value was subtracted from the fluorescence signal of the wells with infected cells. Percent growth inhibition was expressed as the reduction in fluorescence signal in wells incubated with antibody as compared to the negative control.

Plasma Antibody Proteomics

Total IgG was isolated from 1 ml plasma using Protein G Plus Agarose (Thermo #22851) affinity chromatography and cleaved into $F(ab')_2$ fragments using IdeS protease. PfMSP1-specific $F(ab')_2$ was isolated by affinity chromatography using 1 mg recombinant $PfMSP1_{19\text{-}3D7}$ produced in *E. coli* (Meridian Life Sciences, #R01603 and #R01604) coupled to 0.05 mg dry NHS-activated agarose resin (Thermo #26196). This is a large excess of antigen, i.e., a nearly 1:1 molar ratio of antigen to total IgG, of

which only a fraction will bind to $PfMSP1_{19\text{-}3D7}$. $F(ab')_2$ (10 mg/ml in PBS) was rotated at 8 rpm with antigen-conjugated affinity resin for 1 hour at RT, loaded into 0.5 ml spin columns (Thermo #89868), washed 12× with 0.4 ml Dulbecco's PBS (1,000 × *g* for 30 sec. at RT), and eluted with 0.5 ml fractions of 1% formic acid. IgG-containing elution fractions were concentrated to dryness in a speed-vac, resuspended in ddH_2O, combined, neutralized with 1M Tris/3M NaOH. Success of the affinity purification was assessed by confirming the depletion of anti-$MSP1_{19\text{-}3D7}$ $F(ab')_2$ in the flow-through. The sample was then prepared for liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described previously (83, 84) with the modifications that (i) peptide separation using acetonitrile gradient was run for 120 min and (ii) data was collected on an Orbitrap Fusion (Thermo Fisher Scientific) operated at 120,000 resolution using HCD (higher-energy collisional dissociation) in topspeed mode with a 3 sec. cycle time. B cell receptor sequencing data was available from a previous study (43). Demultiplexing of sequence reads and the generation of consensus sequences for UMI groups were performed as outlined by Turchaninova et al. using software tools MIGEC (v1.2.9) and MiTools (v1.5) (85). Sequences with ≥ 2 reads were clustered into clonal lineages defined by 90% HCDR3 amino acid identity using USEARCH (86). LC-MS/MS search databases were prepared as previously described (83), using custom Python scripts (available upon request). MS searches, and MS data analyses were performed as previously described (83, 84), adjusting the stringency of the elution XIC:flowthrough XIC filter to 2:1.

Data Visualization and Statistics

Flow cytometry data were analyzed and plotted using FlowJo (v10.7.1). Dot plots were generated using the package ggplot2 in RStudio (v1.4.1103) using R (v4.0.4). All other plots were generated in GraphPad Prism 9, which was also used for statistical analyses. The statistical test used for each analysis is indicated in the figure legends.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: MassIVE, MSV000088532.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Makerere University School of Medicine Research and Ethics Committee (SOMREC), the University of California, San Francisco Human Research Protection Program & IRB, and the Institutional Review Board of the University of Texas Health Science Center at San Antonio. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EB secured funding for the study, conceived the research question, and designed the study. SG performed flow cytometry. SG, KC, and SB produced recombinant antigens and monoclonal antibodies and contributed to other experiments. GB performed IFA and growth-inhibition assays. SG and RG performed Luminex experiments. RR, RG, and AB performed merozoite ELISAs. KG and GI performed plasma IgG proteomics. IS and BG provided clinical samples and data. SG, SB, and EB wrote the manuscript with input from all other co-authors. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases (R01 AI153425 to EB). SG and AB were supported by Graduate Research in Immunology Program training grant NIH T32 AI138944. RR was supported by Translational Science Training award TL1 TR002647. Data were generated in the Flow Cytometry Shared Resource Facility, which is supported by UT Health, NIH-NCI P30 CA054174-20 (CTRC at UT Health) and UL1 TR001120 (CTSA grant) and in the Genome Sequencing Facility, which is supported by UT Health San Antonio, NIH-NCI P30 CA054174 (Cancer Center at UT Health San Antonio), NIH Shared Instrument grant 1S10OD021805-01 (S10 grant), and CPRIT Core Facility Award (RP160732).

ACKNOWLEDGMENTS

We would like to thank Dr. Kevin O. Saunders (Duke Human Vaccine Institute) for sharing reagents and protocols for the expression of recombinant IgG using linear Ig heavy and light chain gene expression cassettes from Liao et al. The 3T3-msCD40L cell line was a kind gift from Dr. Mark Connors (National Institute of Allergy and Infectious Diseases). Plasmids encoding 3D7 MSP1-bio, MSP3-bio, AMA1-bio, P41-bio, Pf113-bio, Pf3D7_0606800-bio, Pf3D7_1136200-bio, BirA, and rat CD200 were a kind gift from Dr. Gavin Wright (Wellcome Sanger Institute; Addgene plasmids # 47709, 47731, 47741, 47739, 47729, 47790, 47730, 47731, 32408, and 36152). The following reagents were obtained through BEI Resources, NIAID, NIH: *Plasmodium falciparum*, Strain 3D7, MRA-102, contributed by Dr. Daniel J. Carucci; and Monoclonal Antibody N4-1F6 Anti-*Plasmodium falciparum* Apical Membrane Antigen 1 (AMA1) (produced *in vitro*), MRA-481A, contributed by Dr. Carole A. Long.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.809264/full#supplementary-material

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

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frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 03 June 2022
doi: 10.3389/fimmu.2022.866179

Exhausted PD-1$^+$ TOX$^+$ CD8$^+$ T Cells Arise Only in Long-Term Experimental *Trypanosoma cruzi* Infection

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OPEN ACCESS

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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *30 January 2022*
Accepted: *10 May 2022*
Published: *03 June 2022*

Citation:
Gálvez RI and Jacobs T (2022) Exhausted PD-1$^+$ TOX$^+$ CD8$^+$ T Cells Arise Only in Long-Term Experimental Trypanosoma cruzi Infection. Front. Immunol. 13:866179. doi: 10.3389/fimmu.2022.866179

Infection with *Trypanosoma cruzi* remains the most important neglected zoonosis in Latin America. This infection does not lead to specific symptoms in the acute phase, but chronic infection can result in Chagas disease (CD) with cardiac and/or gastrointestinal manifestations that can lead to death. CD8$^+$ T cells are highly effective and essential to control this infection, but fail to eliminate all parasites. In this study, we show that the CD8$^+$ T cells are modulated by the transient induction of co-inhibitory receptors during acute infection of C57BL/6 mice. Therapeutic intervention strategies with blocking antibodies only had a marginal effect on the elimination of parasite reservoirs. Only long-term chronic infection gave rise to dysfunctional CD8$^+$ T cells, which were characterized by high expression of the inhibitory receptor PD-1 and the co-expression of the transcription factor TOX, which plays a crucial role in the maintenance of the exhausted phenotype. PD-1$^+$ TOX$^+$ CD8$^+$ T cells isolated from the site of infection produced significantly less IFN-γ, TNF-α and Granzyme B than their PD-1$^-$ TOX$^-$ CD8$^+$ T cell counterparts after *T. cruzi*-specific stimulation *ex vivo*. Taken together, we provide evidence that, in the context of experimental infection of mice, the magnitude of the CD8$^+$ T cell response in the acute phase is sufficient for parasite control and cannot be further increased by targeting co-inhibitory receptors. In contrast, persistent long-term chronic infection leads to an increase of exhausted T cells within the tissues of persistence. To our knowledge, this is the first description of infection-induced CD8$^+$ T cells with an exhausted phenotype and reduced cytokine production in muscles of *T. cruzi*-infected mice.

Keywords: ***Trypanosoma cruzi*, co-inhibitory receptors, Chagas Disease, T cell exhaustion, TOX, TIM-3, PD-1**

INTRODUCTION

Infection with the obligatory intracellular protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) and Chagas disease (CD) remains an unsolved, ancient affliction in Latin America (LA) (1). It is estimated that more than 8 million people are infected and that 99% do not have access to either diagnosis or treatment (2, 3). Impoverished areas are particularly affected, with more than 65 million people facing the risk of infection every day (4). Migration out of LA has exacerbated CD expansion to other countries, which must now face this new emerging health problem, since

30-35 % of chronically infected cases will develop clinical cardiac or gastrointestinal forms of CD (5, 6). Accordingly, understanding immunity against *T. cruzi* is the key to developing new approaches, such as immune interventions or vaccines, to combat this harmful disease.

Although controlling an acute infection involves the complex interaction of soluble molecules, effector cells of the innate immune system and an adaptive response (7), an adequately regulated $CD8^+$ T cell response is essential to successfully control the replication of the parasite over decades (8). Considering that *T. cruzi* parasites have developed complicated interaction mechanisms with mammalian hosts, there is still limited understanding of two crucial topics: How the parasite evades complete clearance through $CD8^+$ T cells and which specific cellular immune regulatory processes prevent the onset of the disease over time. Similar to chronic viral infections, the persistence of *T. cruzi* parasites throughout the host's lifespan leads to a prolonged effector T cell phase that can drive the cells to functional exhaustion, as is known to occur for anti-viral T cells. In different models of chronic viral infections and tumorigenesis, a hallmark of decreased T cell immunity is the co-expression of co-inhibitory receptors such as Programmed cell death 1 (PD-1), T-cell immunoglobulin mucin-3 (TIM-3), and Lymphocyte-activation gene 3 (LAG-3). Their inhibitory effect is characterized by a progressive loss of function and reduced production of effector cytokines (9–11). Strategies to restore the functionality of $CD8^+$ T cells expressing co-inhibitory receptors involve targeting these receptors using monoclonal antibodies, causing a therapeutic blockade (12, 13). Reversing the state of exhaustion in this manner has led to the development of immunotherapies, specifically "checkpoint Inhibitors", which have been a major breakthrough in cancer treatment until now (14, 15).

Considering the scenario described above, it is conceivable that during the long course of *T. cruzi* infection in humans, T cell exhaustion also takes place. This assumption is supported by a few studies showing dysfunctional T cell responses in other protozoan infections (16, 17), like *Toxoplasma gondii* (18), *Plasmodium* sp (19). and *Leishmania* sp (20, 21). The expression of co-inhibitory receptors during the course of *T. cruzi* infection, their relevance for the elimination of the parasite in the acute phase and their impact on the development of Chagas disease are still controversial. The main reasons are that the acute phase of infection is rarely detected in humans, making it difficult to study big cohorts, and the low number and lack of standardization of experimental mouse models for chronic *T. cruzi* infection (22). Lasso et al. have shown, in a cohort of chronic Chagas patients who had been infected for decades, that $CD8^+$ T cells undergo successive dysfunction during the development of CD. The authors claim that the progressive hierarchical expression of co-inhibitory receptors on the $CD8^+$ T cell compartment and T cell exhaustion are the driving force behind CD (23). The loss of

IFN-γ production by *T. cruzi*-specific $CD8^+$ T cells and correlation with the severity of heart disease was also confirmed by other studies (24, 25). The hypothesis of exhaustion of the $CD8^+$ T cell response due to the long chronic phase is supported by studies of *T. cruzi*-infected children. These children have significantly shorter infection times and their $CD8^+$ T cells show polyfunctional *T. cruzi*-specific responses (26). A series of recent studies in mice highlights disagreements in the field. Pack et al. showed no relevant influence of co-inhibitory receptors on $CD8^+$ T cells: Despite chronic antigen stimulation, $CD8^+$ T cells were able to perform essential functions and contribute to the control of *T. cruzi* parasites in tissue until 100 days post-infection (dpi). The authors concluded that in their model, $CD8^+$ T cell exhaustion does not affect the ability of *T. cruzi* to persist in mice (27). On the other hand, Mateus et al. showed the opposite in their model of chronically infected mice. They describe monofunctional, antigen-specific $CD8^+$ T cells that retain high cytotoxic activity (through the release of Granzyme B (GrB) and perforin) and increased expression of co-inhibitory receptors (mainly CTLA-4 and PD-1) (28). Therefore, whether co-inhibitory receptors on $CD8^+$ T cells play a role in keeping the balance between protection and pathology, and whether *in vivo* blocking of inhibitory signaling pathways can improve the clearance of the parasites and prevent the development of CD, has not yet been fully clarified.

Our investigation focused on the establishment of a mouse model with *T. cruzi* strain Brazil to study the acute and chronic phase of infection, and subsequently characterize the co-inhibitory receptor profile of T cells during infection. Furthermore, we performed a longitudinal analysis of specific T cell responses, cytokine production and serum cytokines to gain deeper insights into the course of infection. Of particular interest was to elucidate whether therapeutic intervention, by blocking highly expressed inhibitory pathways like TIM-3 with monoclonal antibodies, has an immunomodulatory effect during infection and whether this can influence the persistence and thus the development of CD. Since a high percentage of $TIM\text{-}3^+CD8^+$ T cells were identified, therapeutic intervention was conducted at different time points using monoclonal blocking antibodies against TIM-3 to improve the elimination of parasitic reservoirs. We found that only the long-lasting chronic infection (>2 years) gave rise to dysfunctional $CD8^+$ T cells in the tissue of parasite persistence. These $CD8^+$ T cells exhibited high expression of the inhibitory receptor PD-1 and co-expressed the transcription factor TOX, which plays a crucial role in the generation of exhausted $CD8^+$ T cells and the maintenance of their exhausted phenotype. We suggest that these $PD\text{-}1^+$ TOX^+ $CD8^+$ muscle infiltrating lymphocytes might ensure an optimized balance during chronic infection.

MATERIALS AND METHODS

Mice

C57BL/6J and $RAG1^{-/-}$ mice were bred at the animal facility of the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany. Animals were kept in individually ventilated cages, in a temperature-controlled (22 ± 2°C) and pathogen-free animal

facility under biosafety level 3 laboratory conditions (BSL-3). The light/dark cycle was set to a period of 12 hours. Mice received food and water *ad libitum*. Age-matched (7 weeks of age) female mice were used for all experiments.

Cell Line

86HG39 cells were used to keep parasites alive *in vitro*. Cells were maintained at 37°C 5 % CO_2 in complete RPMI (cRPMI, PAN Biotech: 10 % fetal calf serum (FCS), 1% L-glutamine and 0.5 % gentamycin (Capricorn Scientific) and were sub-cultured once a week after 5 min treatment with Trypsin-EDTA (Capricorn Scientific).

Parasites

To recover cryopreserved blood trypomastigotes, the vials were thawed and incubated with a monolayer of 86HG389 cells (29) overnight. Non-infective parasites were removed by collecting cell culture media, the cell monolayer was washed and fresh complete RPMI was added. *T. cruzi*-infected 85HG39 cell culture flasks were maintained at 37°C, 5 % CO_2 until trypomastigotes were released again. The time until 86HG39 cells burst was 6 days for *T. cruzi* Brazil (30).

T. cruzi Infections

To increase the infectivity of the parasites, cell culture-derived trypomastigotes were passaged once in $RAG^{-/-}$ mice. Subsequently, fresh blood of passage mice was collected by heart puncture at the peak of infection using heparinized syringes. Fresh blood diluted in PBS was used to infect the mice groups used for experiments intraperitoneally (i.p.). The infection dose contained 2 x 10^3 trypomastigotes in 200 µl PBS per mouse. Not infected controls (n.i.) only received PBS. All mice groups and controls were infected at the same time under BSL-3 conditions. Mice were controlled daily during the experiments. Endpoint analysis took place on the following days post-infection (dpi): 15, 30 (acute stage) and 60, 100, 250 (early chronic stage) and 800 (late chronic stage). For this, mice were anesthetized by CO_2/O_2 overexposure and sacrificed by cervical dislocation. Organs (heart, liver, skeletal muscle, and spleen) were harvested. Samples were stored in liquid nitrogen until DNA isolation. Spleen and muscle tissue were isolated for flow cytometry. Blood was collected *via* heart puncture for serum analysis.

Analysis of Parasitemia in Blood

To determinate free circulating parasites, 2 µl blood was collected from the tail vein, diluted (1:10) in erythrocyte lysis buffer (10 nM tris pH 7.2, 0.15 M ammonium chloride) and incubated for 5 min. Parasites were counted in a 0.02 mm Neubauer cell chamber.

Quantification of *T. cruzi* Tissue Burden Using Real-Time PCR

Liquid nitrogen-stored tissue samples were thawed and equilibrated to RT. 25 mg were homogenized in 200 µl lysis buffer and proteinase K (Qiagen) in Precellys ceramic Kit tubes and using a Precellys 24 homogenizer (Peqlab, Erlangen, Germany). DNA isolation was performed according to the manufacturer's specifications using a QIAamp DNA Mini Kit (Qiagen). To assess the purity and quantity of DNA we used a NanoDrop® spectrophotometer (Thermofischer). qPCR reactions were prepared using the QuantiTec SYBR Green PCR Kit (Qiagen) and run on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Each reaction was performed in a final volume of 20 µL, containing 50 ng DNA and 0.5 µM each of *T. cruzi* specific primer 121 and 122. Primers target the minicircle variable region from kinetoplast DNA (kDNA) and amplify a 330 bp fragment (31).

121 Fwd: 5′-AAATAATGTACGGGKGAGATGCATGA-3′ and 122 Rev: 5′-GGTTCGATTGGGGTTGGTGTAATATA- 3′. Mouse-specific primers targeting murine GAPDH were used (Fwd: GTCGGTGTGAACGGATTTGG, Rev: TTCCC ATTCTCGGCCTTGAC) (32). Thermal profile as previously published (33). The parasite load was calculated automatically using the Rotor-Gene 6000 Series Software 1.7 (Corbett research/ Qiagen) by plotting the Ct values against standards of known concentration. GAPDH was used to correct the initial DNA sample mount. qPCR standards were generated by spiking tissue homogenates from n.i. mice with cell-cultured *T. cruzi* trypomastigotes. Afterwards, spiked DNA was serially diluted with DNA isolated from n.i. mice tissue. Standards ranged from 10^7 to 10^{-3} (limit of detection; LOD) parasite equivalents per 50 ng of total DNA. A standard curve was generated for each organ.

Preparation of Single-Cell Suspensions From Tissue

Single-cell suspensions of splenic cells were obtained as described in (34). Briefly, spleens were mashed through a 70 µm strainer into cRPMI medium. Erythrocyte lysis was subsequently performed for 5 min at room temperature (RT). Cells were washed with PBS and filtered through a 50 µm strainer. Muscle cells were isolated as described in (35) with some changes. The tissue was dissected from both hindlimbs. Connective and fatty tissue was removed, samples were washed in cold PBS and minced into fine pieces, then digested in 5 ml of digestion medium (0.2% Collagenase type II (2 mg/ml) and 0.05 % Dispase (0.5 mg/ml) in DMEM at 37°C for 40 min. Finally, cells were resuspended in an appropriate volume of PBS and counted.

Flow Cytometry

Antibodies used for flow cytometric analyses are listed in (**Table 1**). Single-cell suspensions were first stained with H-2kb-ANYKFTLV Dextramer (Immundex) in PBS + 2 % FCS for 15 min at RT. Subsequently, surface staining antibodies mixed in Fc-block for surface epitopes were added for 30 min at 4°C. For intracellular cytokine staining, cells were fixed and permeabilized with Foxp3/Transcription Factor Buffer Set (ThermoFisher) for 45 min at RT. After fixation and permeabilization, intracellular staining was performed for 60 min at 4°C. Measurements were performed on a BD Fortessa, a BD LSR II (Beckton Dickinson) Cytometer, and a Cytek Aurora. Flow data were analyzed using the FlowJo 10.8.1 software. Gates were set according to fluorescence minus one (FMO) controls. The gating strategy is depicted in the corresponding **Supplementary Figure**.

TABLE 1 | List of used antibodies.

Marker	Clon	Dilution	Fluorochrome	Manufacturer
CD3	145-2C11	1:200	AF488	Biolegend
		1:300	BUV395	BD BD Invitrogen
		1:300	PE	
		1:200	eFluor 610	
CD3	17A2	1:300	Pe/Cy7	Biolegend
CD4	RM4-5	1:400	V500	Biolegend
CD8	53-6.7	1:200	AF700	Biolegend
CD8	53.67	1:400	V450	eBioscience
CD44	IM7	1:400	PE/Cy7	Biolegend
			AF700	
			BV421	
CD45	30.F11	1:300	APC-Cy7	BD
CD45.1	A20	1:200	AF700	Biolegend
CD45.2	103	1:200	APC/Cy-7	Biolegend
CD62L	Mel-14	1:300	PerCP	Biolegend
			PerCp-Cy5.5	
			Pe	
CD69	H1.2f3	1:100	BV785	Biolegend
CD107a	1D4B	1:200	BV421	Biolegend
LAG-3 (CD223)	C9B7W	1:200	PerCP-Cy5.5	Biolegend
PD-1 (CD279)	RMP1-30	1:200	PE/Cy7	Biolegend
Tim-3 (CD366)	RMT3-23	1:200	APC	Biolegend
			Pe-Cy7	
IFN-γ	XMG1.2	0,3 µL/*well*	AF 488	Biolegend
Granzym B	NGZB	1:200	Pe610	Invitrogen
TNF-α	MP6XT22	1:200	AF700	Biolegend
TOX	REA473	1:100	PE	Miltenyi Biotec
H-2kb- ANYKFTLV Dextramer	—	1:50	PE	Immundex

T. cruzi-Specific *Ex Vivo* Stimulation

Stimulation of *T. cruzi*-specific $CD8^+$ T cells was performed with *T. cruzi* protein lysate from a mix of *T. cruzi* trypomastigotes and amastigotes as described in (26) with some modifications. In brief, cell culture-derived trypomastigotes from the Brazil strain were cultured overnight in Dulbecco's modified Eagle medium (Mediatech; pH 5.0) to transform them into amastigotes. After that, the amastigote and trypomastigote mix was frozen at −196°C and thawed at 56°C four times. After that, the sample was subjected to sonication. The supernatant was collected after centrifugation at 12,000 *x g* and sterile-filtered, then the protein concentration was determined. To stimulate immune cells from the muscle, C57BL/6-Ly5.1 splenocytes were incubated in cRPMI (10 % FCS, 1 % L-Glutamine, 0.5 % Gentamycine) with *T. cruzi* lysate for 5 h. Subsequently, muscle single-cell suspension was added and co-cultured with the antigen-pulsed splenocytes for 5 h at 37°C with 5 % CO_2. To detect the cytokines IFN-γ, TNF-α and GrB, intracellular staining was performed.

Cytokine Analysis

Serum samples were obtained by agglutination for 15-20 minutes at RT in Eppendorf tubes followed by centrifugation for 15 min at 5650 × *g* and stored at −20°C. A custom bead-based Immunoassay LEGENDPLEX™ Murine Cytokine Panel (Customized 13-plex from Biolegend) was performed according to the manufacturer's instructions. Samples were read on a BD Accuri™ C6 Flow Cytometer.

In Vivo Antibody Treatments

TIM-3 neutralization was performed *in vivo* using 100 µg Ultra-LEAF™ (Low Endotoxin, Azide-Free) α-TIM-3 (Biolegend, monoclonal rat IgG) per mouse, injected intra-peritoneally (i.p.) at different time points. Control mice were injected with the corresponding Rat IgG2 a,k (Biolegend).

Statistical Analysis

Statistical significances were analyzed using Graphpad Prism V9 (Graphpad Software, San Diego, USA). Data were analyzed for normal distribution before analysis. All data are shown as mean or mean ± SEM. The statistical significance was determined using a Kruskal–Wallis test with a posthoc Dunn's test. Correlation analysis was conducted using a Pearson Correlation test. Statistical assumptions were made at 95% confidence level and *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. If another test was used, it is indicated in the figure legend.

RESULTS

Kinetics of Parasite Load Confirm that *T. cruzi* Persists Concealed in Skeletal Muscle

To directly evaluate how reliable our experimental infection was and how successful the parasite control is in our mouse model, we analyzed the parasite burden in different organs: Spleen, liver,

heart, and skeletal muscle **(Figure 1).** In line with the results of other groups such as Lewis et. al, obtained using visual techniques (33, 36), our infection resulted in high amounts of parasites disseminated throughout the whole organism during acute infection. Initially, during the acute phase, a high parasite load was recorded in all tissues (15-30 dpi). Subsequently, a strong reduction in parasite load was observed over time, with the lowest parasite burden at 60 and 100 dpi, where it could barely be detected in spleen, liver, heart and muscle. However, the parasite burden increased again at later time points. At 800 dpi, several mice displayed a high parasite burden in the muscle. This suggests that although *T. cruzi* can successfully infect all the organs studied, the skeletal muscle is strongly affected and may serve as a niche for the persistence of the parasites **(Figure 6A).**

Cytokine Expression Profile Reveals a Strong Inflammatory Response Over Time

In terms of immune activation, our infection model is characterized by two completely different phases: The acute and the chronic phase. Accordingly, it is possible to analyze the dynamic nature of cytokine expression during the course of infection in more detail. To this end, we performed a longitudinal analysis of mouse sera from infected mice **(Figure 2)**. As controls, n.i. mice of the same age were analyzed. It is important to emphasize that there was no age-related increase in cytokine expression, as n.i. mice showed no increase in cytokine levels in the sera with increasing age. The pro-inflammatory cytokines IFN-γ, TNF-α and IL-6 were measured as markers for inflammatory processes. During *T. cruzi* infection, IFN-γ was strongly increased over the entire course of the infection. It strongly increased at 15 dpi, during the acute phase, compared to n.i. controls and remained high. The highest levels of IFN-γ were measured in the late chronic phase, at 800 dpi. **(Figure 2A)**. The analysis of TNF-α showed a very similar pattern. It strongly increased during the acute phase of infection, at 15 dpi and 30 dpi, and remained elevated. Like IFN-γ, TNF-α showed a trend to decrease at 60 dpi, 100 dpi and 250 dpi. But, like IFN-γ, the highest levels of TNF-α were measured at 800 dpi, and the differences to n.i. controls were also highly significant at this time point **(Figure 2B)**. TNF-α is a classical marker of chronic inflammatory processes and is associated with the development of heart disease in Chagas patients (37). IL-6 is released after activation of macrophages, but also by neutrophil granulocytes (38). IL-6 is also released by endothelial cells and somatic cells such as fibroblasts after damage (e.g. tissue destruction). In our model, it was markedly elevated during infection **(Figure 2C).** In the acute phase 15 dpi, it was statistically significantly elevated, but not constantly found at this high level, as the concentration fluctuated between different time points. At 60 dpi a scattering was observed: Some animals showed very high levels, while others showed IL-6 levels similar to n.i. control mice. Thus, the difference to the n.i. group is not statistically significant. IL-15 was also analyzed, since it is a pleiotropic cytokine with a broad spectrum of biological functions that can act in an inflammatory or anti-inflammatory manner, depending on the context (39, 40). Interestingly, IL-15 has been reported to be secreted by muscle cells under homeostatic as well as pathological conditions (41, 42) and little is known about its role in *T. cruzi* infection. Examination of serum IL-15 showed a slight increase at 15 dpi compared to n.i. controls, with a trend towards increased levels as the chronic infection progressed. At 100 dpi, the concentration of IL-15 was significantly higher than in the n.i. controls, and IL-15 was also significantly increased in the late chronic phase **(Figure 2D)**. In addition, IL-2 levels were analyzed due to its importance for T cell function by promoting T cell proliferation and differentiation into effector T cells (T_{eff}) as well as ensuring survival and optimal function of T memory and regulatory cells. At 15 dpi, IL-2 was elevated but not significantly different from n.i. controls. It was significantly elevated at 30 dpi, at the end of the acute phase, and remained elevated, though with wide fluctuations, throughout the rest of the chronic infection

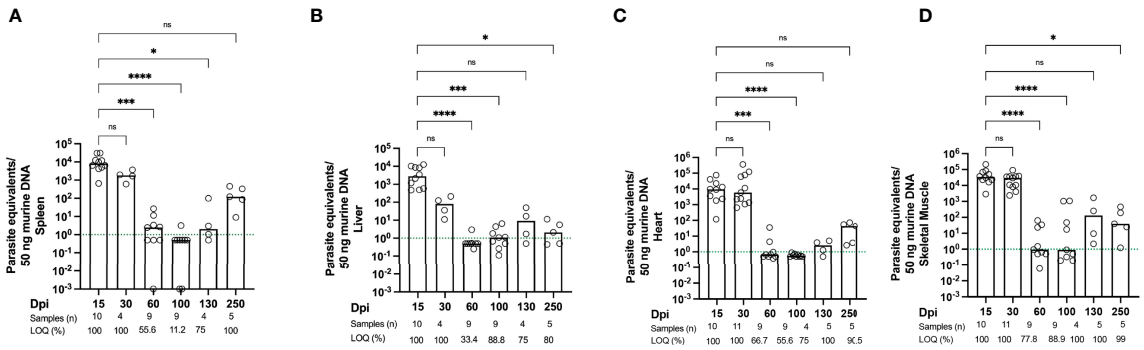

FIGURE 1 | Kinetics of parasite load confirm that *T. cruzi* persists in skeletal muscle. Parasite tissue burden was determined by qPCR. Parasite equivalents per 50 ng DNA were analyzed between 15 dpi (acute phase) and 800 dpi (chronic phase). **(A)** Spleen, **(B)** liver, **(C)** heart and **(D)** sk. muscle (n ≥ 4-23 mice per group). Parasite loads below the limit of quantification (LOQ) were set to LOQ/2, i.e. 0.5 parasite equivalents per 50 ng of DNA. The green dotted line indicates the limit of quantification, which means that samples below this line are still positive, but the parasite load cannot be quantified accurately. 10^{-3} is the limit of detection, as described in Methods. Data were compared using the Kruskal-Wallis test and Dunn's multiple comparison test. All data are representative of 10 independent experiments. Values are presented as mean. *p < 0.05, ***p < 0.001, ****p < 0.0001, n.s. not significant.

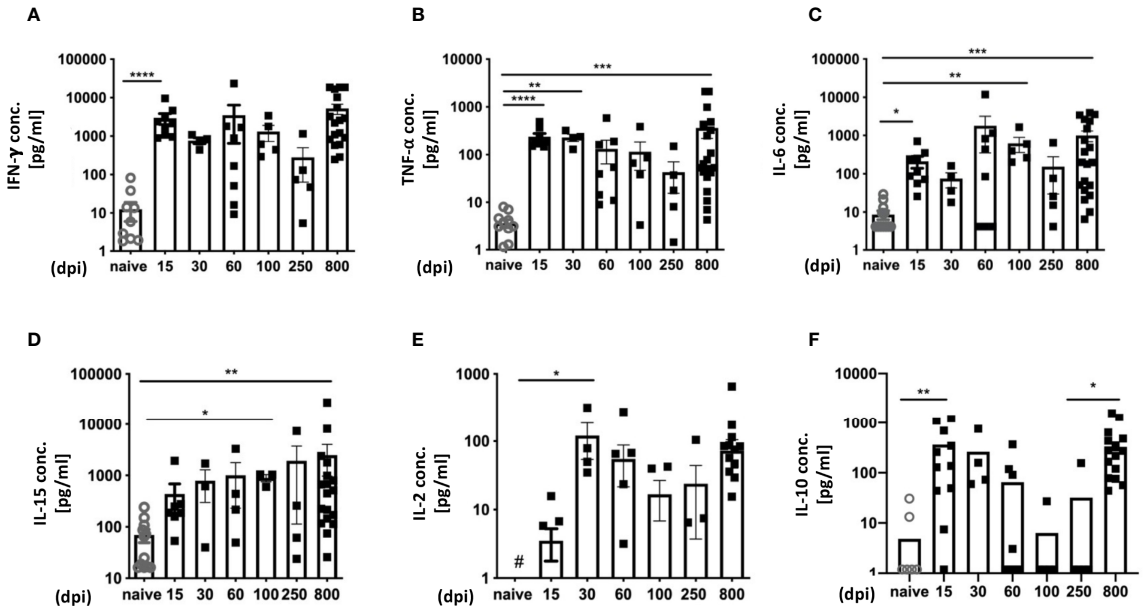

FIGURE 2 | Cytokine expression profiles reveal a strong inflammatory immune response over time. Analysis of cytokines in serum over the course of infection with *T. cruzi* was performed with a bead-based immunoassay to quantify multiple cytokines simultaneously with flow cytometry. The concentrations are given in pg/ml **(A)** for IFN-γ, **(B)** TNF-α, **(C)** IL-6, **(D)** IL-15, **(E)** IL-2 and **(F)** IL-10. A total of n=63 mice were used; n.i. n=13; day 15 n=9, day 30 n=4; day 60 n=8, day 100 n = 4, day 250 = 5 and 800 n=20. Results are presented as mean ± SEM. Data from eight independent experiments were compared using the Kruskal-Wallis test and Dunn's Multiple Comparison Test. # indicates a value below the detection limit of the assay. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(**Figure 2E**). Lastly, IL-10 still plays a controversial role during *T. cruzi* infection; it is an anti-inflammatory cytokine, yet an elevated serum IL- 10 concentration is associated with fibrotic changes and cardiovascular disease (43). Analysis of IL-10 showed a strong and significant increase at 15 dpi compared to n.i. controls (**Figure 2F**). During the course of the infection, IL-10 levels remained elevated until 30 dpi, but from the onset of the chronic phase (60 dpi), there was a decrease in IL-10. The IL-10 concentration continued to decrease in the further course of the chronic phase. However, at 800 dpi a strong increase was measured. In conclusion, these data show that the levels of pro-inflammatory cytokines in particular remained high throughout the whole course of infection in sera of infected mice, despite a strongly reduced parasite burden in the chronic stage.

Induction of Co-inhibitory Receptors on Splenic CD8$^+$ T Cells From Mice With Acute *T. cruzi* Infection

We first determined whether co-inhibitory receptors were induced during experimental *T. cruzi* infection and analyzed the expression of PD-1, LAG-3, and TIM-3 on splenic CD8$^+$ T cells at 15 and 30 dpi using flow cytometry. These time points represent the peak of acute infection, with the highest parasitic load in all analyzed tissues. We focused on the activated effector CD8$^+$ T cell population, characterized as CD44$^+$ CD62L$^-$, since polyfunctionality of this subset is necessary to limit further parasite dissemination. The gating strategy and representative dot plots are depicted in **Supplementary Figures 1A–E**. Splenic CD8$^+$ T cells showed a significant increase in the expression of all three markers compared to not infected controls (n.i.). Our results revealed a strong and statistically significant increase in the percentage of TIM-3$^+$ T cells (**Figure 3A**). This molecule is an important regulator of T cell-mediated immune responses and its expression correlates with the strength of activation by the T cell receptor. The n.i. controls showed very low expression of this molecule, with on average 0.65 % of CD8$^+$ T cells in the spleen being TIM-3$^+$. The TIM-3$^+$ population decreased towards the end of the acute phase at 30 dpi, but was still statistically significantly higher than in n.i. controls. The frequency of the PD-1$^+$ population also increased in comparison to n.i. controls, but this increase was lower than the increase in TIM-3 (**Figure 3B**). These results are in contrast to earlier findings in other infection models, in which strong T cell activation leads to a high transient PD-1 expression (44). LAG-3 also showed increased expression compared to not infected controls (n.i.), which was significant at 15 dpi and also 30 dpi (**Figure 3C**). The question arose whether there was co-expression between TIM-3, PD-1 and LAG-3. The proportion of co-expression of these markers within effector CD8$^+$ T cells is depicted for 15 dpi in **Figure 3D** and for 30 dpi in **Figure 3E**. In summary, the analysis of CD8$^+$ T cells showed that strongly activated CD8$^+$ T cells in the spleen transiently expressed the co-inhibitory receptors TIM-3, PD-1 and LAG-3. After the establishment of the early chronic *T. cruzi* infection, when the parasitemia was largely controlled, none of these co-inhibitory receptors could be detected as phenotypic markers for T-cell exhaustion until

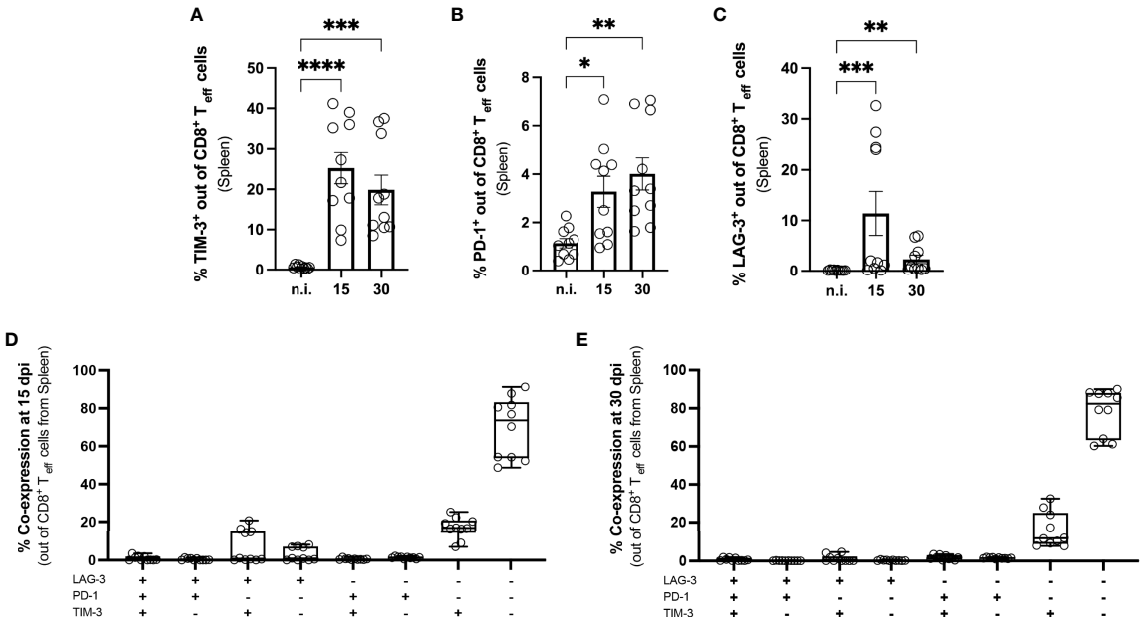

FIGURE 3 | Induction of co-inhibitory receptors on splenic CD8$^+$ T cells from mice with acute *T. cruzi* infection. Flow cytometric analysis of splenic CD8$^+$ T cells at 15 and 30 dpi. The gating strategy is depicted in S. 1 **(A)** Effector CD8$^+$ T cells were defined as CD44$^+$ CD62L$^-$. Results are presented as percentages of effector CD8$^+$ T cells **(A)** for TIM-3, **(B)** for PD-1 and **(C)** for LAG-3. A boolean gating analysis was performed to combine these three markers **(D)** for 15 dpi and **(E)** for 30 dpi. Data are from three independent experiments. Data in **(A–C)** were compared using the Kruskal-Wallis test and Dunn ís multiple comparison test. Results are presented as mean ± SEM (n.i. n = 10; infected day 15 n = 10; day 30 n = 10). *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.

250 dpi (**Supplementary Figures 1F–H**). Interestingly, in the late chronic stage at 800 dpi, PD-1$^+$ T cells were once again present in spleen and muscle (**Supplementary Figure 1G**).

High Frequency of TIM-3$^+$ CD8$^+$ T Cells in the Muscle of Mice With Acute *T. cruzi* Infection

To investigate the influence of the infection on the CD8$^+$ T cells in the muscle, tissue isolated at 15 and 30 dpi was enzymatically digested. The immune infiltrate in the muscle was characterized by flow cytometry (**Figures 4A–D**). In infected mice, the muscle contained a high number of CD3$^+$CD45$^+$CD8$^+$ T cells compared to the muscle of n.i. mice. CD8$^+$ T cells in the muscle of infected mice were highly activated and CD44$^+$CD62L$^-$ (**Supplementary Figure 2**), similar to the results for the spleen. They also expressed the co-inhibitory receptor TIM-3 to a higher extent. The highest frequency of TIM-3$^+$ CD8$^+$ T cells was found at 15 dpi and 30 dpi, decreasing at later time points (**Supplementary Figure 1F**). Since only a small percentage of CD8$^+$ T cells were PD-1$^+$ and the *T. cruzi* infection did not affect the frequency of

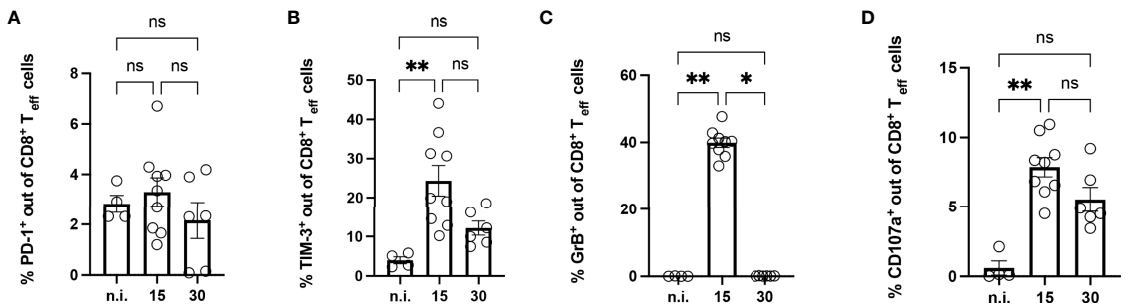

FIGURE 4 | Analysis of CD8$^+$ T cells isolated from the muscle of mice with acute *T. cruzi* infection. Phenotypical analysis of CD8$^+$ T cells isolated from muscle tissue was performed at 15 and 30 dpi using flow cytometry. Results are presented as percentages of effector CD8$^+$ T cells **(A)** for PD-1, **(B)** for TIM-3, **(C)** for GrB, and **(D)** for CD107a. GrB and CD107a were stained *ex vivo* without previous stimulation. GrB was stained intracellularly. Results are presented as mean ± SEM. Data from three independent experiments were analyzed using the Kruskal-Wallis test and Dunn ís multiple comparison tests (n.i. n=4, day 15 n=9, and day 30 n=6). *p < 0.05, **p < 0.01, n.s. not significant.

PD-1$^+$ CD8$^+$ T cells (**Figure 4A**), we focused on the analysis of TIM-3$^+$CD8$^+$ T cells (**Figure 4B**). To determine the cytotoxic potential of effector CD8$^+$ T cells from muscle tissue, the intracellular expression of GrB and CD107a was determined (**Figures 4C, D**). CD107a, which is exposed on the cell surface as a result of the degranulation of CD8$^+$ T cells, serves as a marker for cytotoxicity. While the CD8$^+$ T cells of the n.i. mice expressed neither GrB nor CD107a, the frequency of cells expressing these markers was highly increased in infected mice at 15 dpi. It should be emphasized that GrB could no longer be detected at 30 dpi. This loss of GrB production at 30 dpi was also recently described by *Mateus et al.* in spleen cells from *T. cruzi*-infected mice (28). In contrast, analysis of CD107a showed consistently higher levels than the n.i. control without being statistically significant at 30 dpi. It should be noted that the strongest CD107a expression was found in TIM-3$^+$CD8$^+$ T cells at 15 dpi (not shown). Even if no GrB could be detected, the CD107a expression suggests that degranulation had occurred at 30 dpi.

Blockade of TIM-3 Did Not Improve Parasitic Clearance in the Muscle

Based on the results of the previous experiments, we next aimed to investigate the effect of a therapeutic blockade of TIM-3 using α-TIM-3 during the peak of TIM-3 expression. The expected outcome, as shown in other infection models (45, 46), was an enhanced T cell response, thus more effective elimination of the parasite (47). For this reason, reduction of the parasite load in the tissue was used as a parameter to evaluate the effect. A scheme for the administration of antibodies is shown in (**Figure 5A**). Tissue samples from the spleen, muscle, heart and liver were collected to determine the parasite load. The results of the qPCR for the spleen and muscle are shown (**Figure 5B**). The TIM-3 blockade only resulted in a trend towards a reduction of parasitic burden in muscle tissue, but this difference was not statistically significant compared to the control isotype antibody-treated group. Thus, we could not demonstrate that blocking TIM-3 has an effect on eliminating the parasite in the muscle reservoir. Furthermore, the parasitic load in the spleen was highly increased. Heart and liver tissue did not show differences in the parasitic burden as a consequence of the α-TIM-3 treatment **(data not shown).**

Loss of Parasite Control in Muscle Tissue in the Late Chronic Phase

Muscle and heart samples were isolated and the parasitic burden was determined by qPCR. In the heart tissue, many samples exhibited a parasite burden below the quantification limit of the qPCR. The mean parasite burden determined for the heart was 3.5 parasites/50 ng heart tissue, while in muscle 8800 parasites/50 ng muscle tissue were found. Comparing the parasite burden in the heart and muscle tissue for each mouse **(Figure 6A)**, the parasite burden is significantly higher in muscle tissue than heart tissue, and there is no correlation between the number of parasites in muscle and the number of parasites in the heart of the same mouse. Furthermore, a large scattering of the values can be seen, ranging from 1 to 1x10^6 parasites/50 ng DNA in muscle tissue. This number of parasite equivalents per nanogram tissue corresponded to the observed tissue parasite burden in the acute phase **(Figure 1)**. This strongly suggests that, although all mice were in good health, some mice in the infection group were less able to control tissue parasite burden in the skeletal muscle over a longer period. The parasite load and the number of activated CD8$^+$ T cells present in the muscle were correlated and the r-value of 0.54 indicates a moderate positive correlation between the parasite burden and the number of activated CD8$^+$ T cells in the muscle **(Figure 6B)**.

Prolonged Infection Leads to Expression of PD-1 and TOX of CD8$^+$ T Cells in the Muscle

Next, the T cells from the muscle were further examined by flow cytometry. Representative plots and the gating strategy are

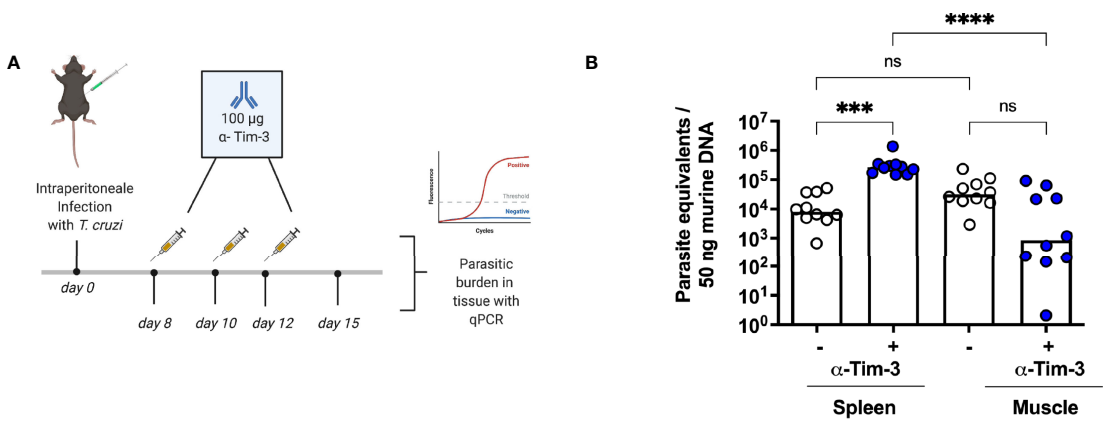

FIGURE 5 | A blockade of TIM-3 increased parasitemia in the spleen but did not improve clearance in the muscle. A blockade using α-TIM-3 was performed. **(A)** Application scheme for the α-TIM-3 treatment. The control group received an isotype antibody using the same regimen. **(B)** The effect of TIM-3 blockade on parasitic load in tissues was analyzed by qPCR. Results are presented as mean. Data from two independent experiments were analyzed using the Kruskal-Wallis test and Dunn ís multiple comparison test (infected mice with α-TIM-3 n = 10, infected mice with isotype antibody n = 10). ***p < 0.001, ****p < 0.0001, n.s. not significant.

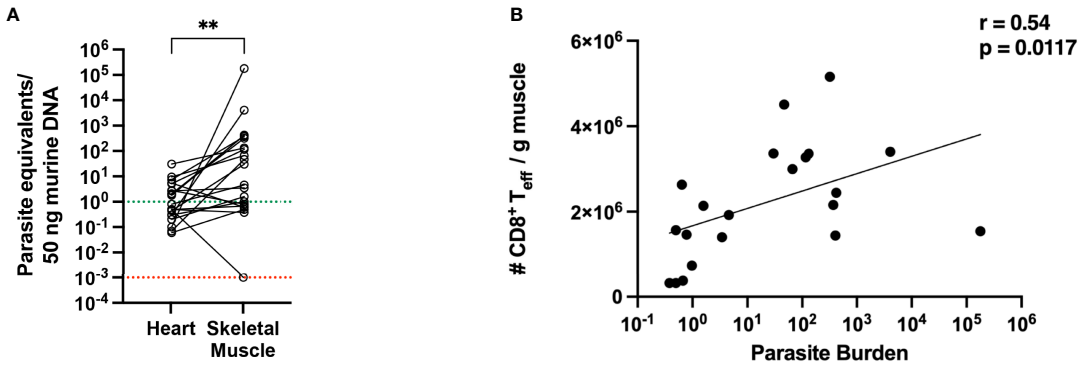

FIGURE 6 | Parasitic burden in muscle tissue is higher than in heart tissue and shows a positive correlation with the number of activated $CD8^+$ T cells. **(A)** Parasite equivalents per 50 ng DNA were analyzed from heart and muscle tissues at 800 dpi of infection by qPCR. Data were pooled from two independent experiments (total infected animals n = 20). Results were analyzed for statistical differences with a two-tailored Mann-Whitney test. **(B)** Pearson correlation between parasitic burden in the muscle and the absolute numbers of activated effector $CD8^+$ T cells at 800 dpi. **p < 0.01.

depicted in **Supplementary Figures 3A, B**. In infected mice, a higher infiltration of $CD3^+$ T cells could be observed (**Figure 7A**). The biggest fraction of infiltrating cells was $CD8^+$ T cells (**Figures 7B–D**). Using MHC-I dextramer staining against the immunodominant $TSKB20^+$ epitope of the *T. cruzi trans*-sialidase, we also found a strong enrichment of these cells (**Figure 7E**). $CD44^+CD62L^-$ effector $CD8^+$ T cells were examined for the expression of activation markers and co-inhibitory receptors. The frequency of effector cells was significatively increased in infected mice (**Supplementary Figure 4A**). At this late timepoint, neither TIM-3, LAG-3 or GrB could be detected on $CD8^+$ T cells from the muscle of infected mice anymore (**Supplementary Figures 4B–D**). CD69 is a marker for early T cell activation, but it also marks non-circulating tissue-resident cells in non-lymphoid tissues (48–50). The percentage of $CD69^+$ T cells was significantly higher than in

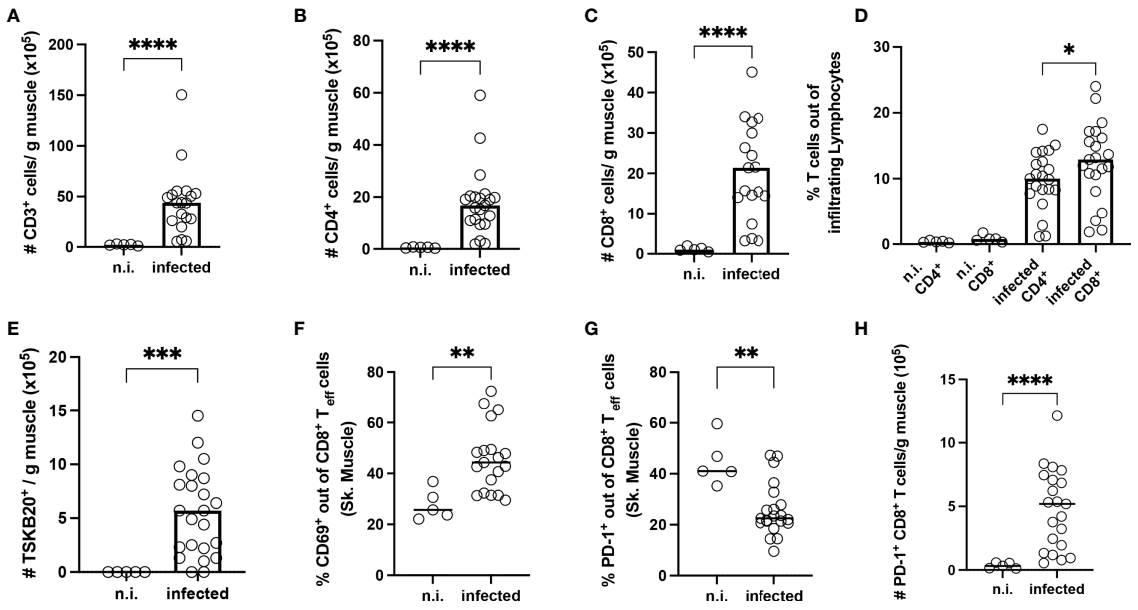

FIGURE 7 | Long-term infection with *T. cruzi* leads to strong infiltration of T cells into the skeletal muscle tissue. T cells were isolated from the skeletal muscle of infected mice at 800 dpi and analyzed by flow cytometry. Upper line **(A–C)** Results expressed as absolute numbers per gram of muscle tissue. **(A)** $CD3^+$, **(B)** $CD4^+$ and **(C)** $CD8^+$ T cells. **(D)** Frequency analysis of infiltrating T cells. Bottom line (E-H): In absolute numbers **(E)** $TSKB20^+$ T cells. **(F, G)** Results expressed as a percentage of effector $CD8^+$ T cells (T_{eff}). T_{eff} population was defined as $CD44^+CD62L$. A representative plot of the staining and gating strategy is depicted in S. 3 A-B. In **(F)** CD69, and in **(G)** PD-1. Absolute numbers of PD-1 cells **(H)**. Data are from two independent experiments. Results are presented as mean (n.i. n = 5; infected n = 20) and analyzed by the Kruskal-Wallis test and then by Dunn's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

n.i. controls **(Figure 7F)**. Additionally, comparing T_{eff} cells from the same mice between the tissues, although the absolute numbers of $CD8^+$ T cells in the spleen were comparable to the numbers found in the muscle **(Supplementary Figure 4E)**, the frequency of $CD69^+CD8^+$ T cells was significantly increased only in T_{eff} cells from muscle tissue, whereas T_{eff} cells from the spleen expressed much less CD69 **(Supplementary Figure 4F)**. In addition, there is a moderate negative correlation between the absolute number of $CD8^+$ T cells between the spleen and the muscle, suggesting an accumulation of $CD8^+$ T cells in the muscle over time **(Supplementary Figure 4G)**. While analyzing the PD-1 expression on the isolated $CD8^+$ T cells, it became apparent that a high percentage of the very few $CD8^+$ T cells found in the muscle of n.i. mice already expressed PD-1 **(Figure 7G).** However, upon infection, the absolute numbers of $CD8^+$ T cells strongly increased **(Figure 7H)**. Thus, upon infection, an increased absolute number of $PD\text{-}1^+CD8^+$ T cells was found in the muscle.

In conclusion, a strong accumulation of $PD\text{-}1^+CD8^+$ T_{eff} in the muscle was only found after a very long infection period of two years. The high frequency of $CD69^+$ $CD8^+$ T cells within the muscle, compared to the very low frequency of these cells in the spleen, presents an argument against CD69 induction only as a consequence of an ongoing T cell activation, and supports a tissue-resident phenotype in the muscle. Strikingly, $CD8^+$ T cells in the muscle directed against the immunodominant TSKB20 epitope derived from the *trans*-sialidase of *T. cruzi* remained $PD\text{-}1^-$ (**Supplementary Figure 5A**).

Effector $PD\text{-}1^+$ $CD8^+$ T Cells Co-Express the Transcription Factor TOX and Show a Reduced Capacity to Produce Cytokines After *Ex Vivo* Stimulation

To further explore whether the $PD\text{-}1^+$ T cells were exhausted, the expression of the transcription factor TOX was analyzed. The frequency of $PD\text{-}1^+$ TOX^+ $CD8^+$ T_{eff} was significantly increased in infected mice **(Figure 8A)**. A representative staining of PD-1 and TOX gated on $CD8^+$ T_{eff} cells showing clear co-expression is depicted in **Supplementary Figure 5B**. To test the capacity of $PD\text{-}1^+$ $CD8^+$ T_{eff} cells to produce cytokines, these cells were stimulated *ex vivo*. The gating strategy and representative dot plots are depicted in **Supplementary Figure 6**. Lymphocytes from the muscle of infected mice were co-cultivated with spleen cells isolated from n.i. Thy1.1-congenic C57BL/6 mice. These cells were pre-incubated with *T. cruzi* lysate, since cells from the spleen contain an appropriate number of antigen-presenting cells (APC) to better stimulate the T cells isolated from the muscle. This experimental set-up takes advantage of the fact that the cells from Thy1.1 C57BL/6 mice express the congenic marker CD45.1 **(Supplementary Figure 6B)**, whereas cells from a wild-type C57BL/6 mouse express the CD45.2 allele **(Supplementary Figure 6C)**. Thus, n.i. splenic cells used as APC were $CD45.1^+$ and could be discriminated from $CD45.2^+CD8^+$ T cells from muscle tissue of infected mice. The ability of $PD\text{-}1^+$ TOX^+ T cells to produce pro-inflammatory cytokines and cytotoxic granules after stimulation was examined. The gating strategy and representative dot plots for this staining are depicted in **Supplementary Figure 6D**. Production of IFN-γ, TNF-α and GrB was found to be impaired **(Figures 8B–D).**

In summary, the experiments in the late chronic phase showed that a very long infection period can indeed lead to an accumulation of dysfunctional $CD8^+$ T cells, which are characterized by a high degree of co-expression of the co-inhibitory molecule PD-1 and the transcription factor TOX. TOX, therefore, appears to play a crucial role in generating and maintaining the exhausted phenotype of $CD8^+$ T cells in this model. Nevertheless, the $CD44^+CD62L^-CD69^+PD\text{-}1^+TOX^+$ T cells infiltrating the muscle still appear to exert control over the parasite burden in the tissue. However, whether this represents an optimal equilibrium that occurs in the course of chronic infection or represents the beginning of a complete loss of pathogen control must be clarified in further studies.

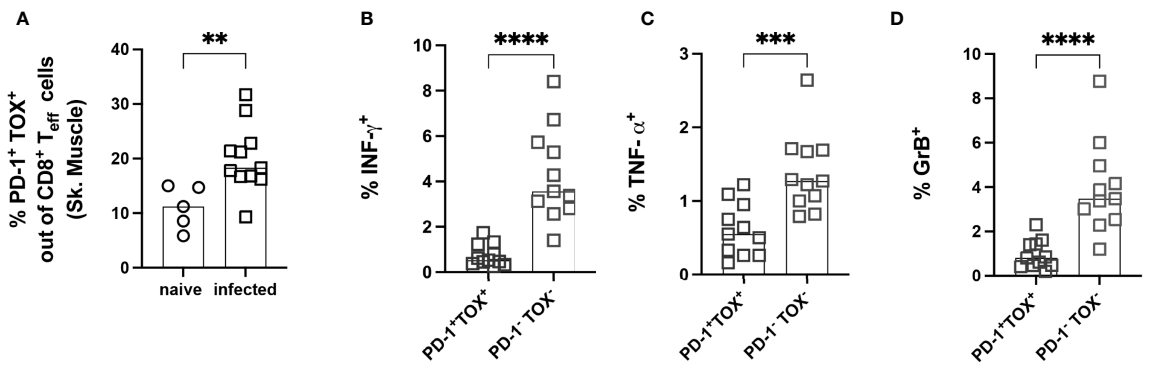

FIGURE 8 | Effector $PD\text{-}1^+$ $CD8^+$ T cells co-express the transcription factor TOX and show a reduced capacity to produce cytokines after *ex vivo* stimulation. Functional analysis of $PD\text{-}1^+$ TOX^+ $CD8^+$ T_{eff} from muscle tissue. Representative staining for the co-expression of PD-1 and TOX on $CD8^+$ T_{eff} cells is depicted in **Supplementary Figure 5B**. **(A)** Co-expression of PD-1 and TOX was quantified and expressed as the frequency of $CD8^+$ T_{eff} cells. $CD8^+$ T_{eff} cells were stimulated with *T. cruzi* lysate to investigate functionality. IFN-γ **(B)**, TNF-α **(C)** and GrB **(D)** were stained intracellularly. Data are from one experiment and were compared using the Kruskal-Wallis test and Dunn's Multiple Comparison Test. The results are presented as mean (n.i. = 5, infected n= 11). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

DISCUSSION

The intracellular protozoan parasite *T. cruzi* is a complex, ancient eukaryotic organism and a versatile pathogen that can persist in the host organism for life. The *T. cruzi* host-parasite interaction is immunologically highly interesting, as the control of the infection is mainly ensured by $CD8^+$ T cells. In models of chronic viral infections, it has already been shown that T cells undergo phenotypic changes characterized by a progressive increase and co-expression of co-inhibitory receptors, leading to exhaustion and dysfunctionality. This means that important T cell functions such as cytotoxicity (release of granules and GrB), proliferation and the production of cytokines such as IFN-γ, TNF-α and IL-2 are diminished or even lost (51). Although there is no definitive definition and marker combination to describe exhausted T cells, co-expression of PD-1, LAG-3 and TIM-3 has been extensively described (52, 53). To prevent permanent damage to the host due to the immune response, $CD8^+$ T cells must be negatively regulated in their function to ensure immune homeostasis (54). This study aimed to characterize the co-inhibitory molecule profile of $CD8^+$ T cells during infection with T. *cruzi* in detail, and to test whether the co-expression of multiple co-inhibitory receptors on $CD8^+$ T cells, as a physiological mechanism to avoid immunopathology, favors parasite persistence and disease. To this end, a model was established based on the intra-peritoneal infection of female C57BL/6 mice with T. *cruzi* trypomastigotes of the strain Brazil (DTU I) (55, 56). The acute infection with *T. cruzi* Brazil was characterized by a ubiquitously high parasite load while remaining asymptomatic, as found in humans. This means that these mice did not show severe weight change or signs of acute disease. The parasitemia was successfully controlled within 30 days and led to a highly restricted distribution of parasites in the muscle tissue as a reservoir during the chronic phase. For a long time, tissue preferences leading to a heterogeneous load in different tissue types was described as a characteristic of many *T. cruzi* strains. For example, some strains have been described as myotropic and others as reticulotropic (57). In 2014, the development of highly sensitive bioluminescence imaging techniques by Lewis et al. demonstrated that, regardless of the discrete typing units (DTU) affiliation of the *T. cruzi* strain, persistence is mainly restricted to skeletal muscle (33, 58). Our results reproduce the infection courses of other research groups (27, 59). The late chronic infection at 800 dpi was characterized by loss of parasite control in some mice. Taken together, the mouse model established for this study reliably reproduces the infection dynamics described with other techniques and is suitable to investigate the mechanisms leading to persistent infection, which is the most important question in CD research. In this study, it was clearly shown that $CD8^+$ T cells from the spleen and muscle are highly effective during the acute phase of infection and are not characterized by the expression of multiple co-inhibitory receptors. First, the results confirm that the T cell response in the infected mice is effective enough to ensure survival and that the $CD8^+$ T cell response is concentrated on the immunodominant epitope TSKB20 of the *trans*-sialidase (60). In addition, we found an expression of individual co-inhibitory receptors such as TIM-3 and to a lesser extent of PD-1 and LAG-3 on $CD8^+$ T cells. The transient expression of PD-1 on $CD8^+$ T cells may also be interpreted as a consequence of their activation (44). LAG-3 was particularly strongly induced at 15 dpi. LAG-3 expression might indicate the involvement of type 1 regulatory T cells (Tr1) (61). These cells have been characterized by LAG-3 and CD49b expression and confer suppressive capacity. However, we could not find T cells with the aforementioned phenotype at any time point during infection. However, it should be noted, that we cannot exclude that $CD8^+$ T cells expressing LAG-3 and TIM-3, as found in our study, could have also a suppressive capacity as recently described by Brandi et al. using an acute mouse model of malaria (62). $CD8^+$ T cells with a similar co-inhibitory-rich phenotype could also be found in patients suffering from acute malaria further highlighting their relevance in acute infections. In our model, TIM-3 was the co-inhibitory molecule that was most strongly induced on $CD8^+$ T cells upon infection. TIM-3 was first described on differentiated IFN-γ producing $CD4^+$ and $CD8^+$ T cells, and induced T cell apoptosis by binding to galectin-9 (63, 64). TIM-3 has also been shown to mark the most exhausted and dysfunctional T cells that arise in chronic viral infections such as HIV or in cancer (9, 65). However, TIM-3 expression, in an acute state, can also be the result of a strong activation (66–68). Avery et al. have shown that TIM-3 partially exerts a costimulatory function by amplifying signals in the immunological synapse. It is thought that excessive activation by the TCR and an excessive effector $CD8^+$ T cell response disrupt the formation of T cell memory, and that this is how TIM-3 contributes to T cell exhaustion (69). Although blockade of TIM-3 has been shown to enhance the T cell response and restore its functionality in other models (12, 70, 71), in this study the blockade of this molecule in the acute phase of *T. cruzi* infection only resulted in a non-significant change in parasite load in the muscle. The fact that the TIM-3 blockade led to an increase in tissue burden in the spleen may be due to the effects that the TIM-3 blockade exerts on cells of the innate immune system. TIM-3 is expressed on many cells of the innate and adaptive immune system, particularly on macrophages, for which TIM-3 blockade has been shown to increase their number and activation (72). For this reason, it is conceivable that the TIM-3 blockade would allow highly activated macrophages, which have already eliminated many parasites, to accumulate in the spleen. Thus, detection of tissue load by qPCR after blockade could indicate a higher load even though these parasites are no longer vital. Further experiments are needed to verify this hypothesis, since assessing the effects of TIM-3 blockade on other immune cells was not within the scope of this work. The decrease in PD-1 and TIM-3 at the end of the acute phase (30 dpi) can be interpreted as a sign of a resolution of the T cell response. During the transition from the acute to the chronic phase, the amount of antigen decreases sharply, as the chronic infection is associated with very low parasitemia. The results of our study cannot support the hypothesis that

complete elimination of *T. cruzi* is prevented due to T cell exhaustion or even an over-regulated T cell response, since $CD8^+$ T cells were not inhibited in their effector function during the acute phase of infection, as indicated by the successful control of parasite proliferation. Thus, the persistence of *T. cruzi* in infected mice is not the result of excessive negative regulation or dysfunctional $CD8^+$ T cells. The data shown here provide no evidence for a limitation of $CD8^+$ T cell function due to co-inhibitory receptors expressed during the acute phase of *T. cruzi* infection. The study of the early chronic phase showed that $CD8^+$ T cells are crucial for the long-term control of *T. cruzi* infection. $CD8^+$ T cells showed no expression of co-inhibitory receptors in chronically infected mice. Studying $CD8^+$ T cells in the muscle, being the tissue of persistence, is highly relevant, as it is the site where antigen exposure is highest for an extended period of time, which could contribute significantly to T cell exhaustion. During long-term infection, a population of activated effector $CD44^+CD62L^-$ $CD8^+$ T cells massively infiltrate the muscle and may continuously control the spread of the parasites. This study shows, for the first time, that a proportion of these effector $CD8^+$ T cells in the muscle co-express PD-1 and the transcription factor TOX. TOX is a recently described transcription factor that epigenetically determines T cell exhaustion (73, 74). In line with this, after antigen-specific restimulation, effector $PD\text{-}1^+TOX^+CD8^+$ T cells exhibited a marked loss of effector functions, producing significantly less IFN-γ, TNF-α and GrB compared to their $PD\text{-}1^-TOX^-$ counterparts. Thus, the emergence of these T cells with an exhausted phenotype is the result of parasite persistence rather than its cause. This hypothesis is strongly supported by recent studies showing that an increase in MHC-I expression in mouse muscle leads to an enhanced $CD8^+$ T cell-mediated reduction in parasite burden. However, prolonged overexpression of MHC-I resulted in a loss of parasite control and a massive increase in exhausted $PD\text{-}1^+CD8^+$ T cells (75). It is noteworthy that $CD8^+$ T cells recognizing an immunodominant epitope of the *T. cruzi trans*-sialidase were also found in the muscle, but, in contrast to other $CD8^+$ T_{eff}, they did not express PD-1 at all. The reason for this dichotomy remained unclear, but it is tempting to speculate that the lack of PD-1 expression might contribute to their immunodominance. However, it has already been shown that these *trans*-sialidase-specific $CD8^+$ T cells did not contribute to protection, despite their high number (76, 77). The data shown here prove that a very long chronic infection with *T. cruzi*, even in the mouse model, can lead to a loss of function of $CD8^+$ T cells. Moreover, although these T cells express only one of the co-inhibitory receptors analyzed here, namely PD-1, the co-expression of TOX marks them as exhausted cells. Alternatively, they may have adapted to the situation of chronic infection and only retained the necessary functionality to control the pathogen without harming the host (54).

During the course of the entire infection, there was a strong systemic inflammation, which was evident from the examination of cytokines in the serum, even though the chronic infection established in this mouse model followed an asymptomatic course. Modeling Chagas disease using mice remains a challenge, but it is nevertheless essential to identify factors leading to pathology. It remains unclear whether the very long chronic courses of infection seen in humans (30 years), leading to a deterioration of the immune response, can be reproduced during the shorter lifespan of mice (78). One limitation of this study is that we did not address sex-specific differences, although sex might influence the course of infection or the development of heart disease. There is no clear evidence of sex-specific differences in Chagas disease. However, it has been shown that there are increased pathological changes, including myocardial damage in male mice (79) and that female mice are more resistant to *T. cruzi* infection than male mice (80). In addition to sex differences, studies have shown a high prevalence of comorbidities in Chagas patients, probably due to advanced age (81). The infection with *T. cruzi* triggered a strong systemic induction of pro-inflammatory cytokines. IFN-γ, which remained elevated throughout the course of the infection, plays a central role in combating *T. cruzi*. In IFN-γ deficient mice, a *T. cruzi* infection, even with a low-virulence strain, is lethal (82). Increased IFN-γ polarizes the immune response towards differentiation into T_H1 T cells. It plays a major role in the defense against intracellular pathogens by activating macrophages and differentiating cytotoxic $CD8^+$ T cells, which are essential for the elimination of *T. cruzi*. Macrophages increase antigen presentation, produce nitric oxide and reactive oxygen radicals as well as nitrogen-dependent microbicidal defense systems. Finally, macrophages activated by IFN-γ produce more TNF-α. A robust immune response by IFN-γ-producing $CD4^+$ T cells is necessary to control parasite replication, and loss of this cytokine also leads to a loss of these protective mechanisms. This has been shown in studies of Chagas patients who have an HIV co-infection or are immunosuppressed (83, 84). The pathological effects of chronically elevated IFN-γ levels, as found in infected muscle tissues, are also still unclear. IFN-γ-induced cytokine-mediated inflammation has been observed in Chagas patients and has been connected to cardiac pathology (85, 86). TNF-α is mainly secreted by activated monocytes and macrophages, and also by T_H1 helper cells and $CD8^+$ cytotoxic T cells in small amounts. It leads to strong endothelial activation by inducing adhesion molecules and intensifying local inflammation. In many chronic inflammatory states, TNF-α induces cachexia, i.e., loss of muscle mass through catabolic processes. Patients with high levels of IFN-γ and TNF-α were found to have a higher risk of disease progression (87, 88). The most interesting cytokine in the context of *T. cruzi* infection in this study was IL-15, which is also strongly induced by the infection and remains at a very high level throughout the course of the infection. The source of the large amounts of IL-15 remains unclear. IL-15 fulfills several functions, such as supporting the survival of T cells and the activation of NK cells, both of which are essential cell types for a robust immune response against *T. cruzi* (40, 89). A recent study by Wu et al. has shown that $CD8^+$ T cells are functionally impaired or depleted in chronic viral infections and that this phenomenon is accompanied by an unwanted loss of muscle

mass. The production of IL-15 by muscle tissue has already been described (41), however, the authors show that the production of muscle IL-15 regulates T cell exhaustion. IL-15 produced by muscle tissue supports the recruitment of $CD8^+$ T cells within the muscle microenvironment, and this protects them from overwhelming activation during inflammation. Wu et al. showed that the $CD8^+$ T cells in muscle have a higher proliferative potential than T cells in other secondary lymphoid organs, such as the spleen. When required, T cells from the muscle re-enter the lymphoid tissue and differentiate into functional effector T cells. The authors postulate that muscle-specific IL-15 production counteracts T cell exhaustion by protecting the proliferative potential of T cells from inflammation, and by protecting the T cell compartment in lymphoid organs (42). In our model of *T. cruzi* infection, T cell exhaustion occurs very late. At early time points $CD8^+$ T cells are fully functional and control parasite replication. However, residual parasite burden in specific niches like the muscle were not cleared leading to a continuous presence of antigen. It remained to be studied based on the results of this work, if the muscle is the source of the elevated IL-15 levels in the serum which might delay exhaustion of $CD8^+$ T cells despite chronic antigen exposure and if this also counteracts atrophy of the muscle induced by TNF-α as described by O´Leary et al. (90).

Overall, the model established in this study is excellent to investigate parasite persistence mechanisms in the muscle, whereas the pathological processes in the heart do not yet correspond to those in humans. The persistence does not seem to be the result of $CD8^+$ T cells dysfunction, which can control parasitemia over a long period very efficiently. However, a few parasites in the muscle evade this control for as yet unexplained reasons. Deciphering these mechanisms holds the potential for new immunotherapies to be developed in order to further reduce the number of persistent parasites in the muscle, or even eliminate them completely.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Federal Authority of the State of Hamburg (Hamburger Behörde für Justiz und Verbraucherschutz, Antrag 52/17).

AUTHOR CONTRIBUTIONS

TJ conceived and supervised the study; RG conceived and conducted all mouse experiments, collected and analyzed data and wrote the manuscript. Both authors revised the manuscript and approved the submitted version.

FUNDING

RIG was supported by a merit-based scholarship from the Konrad Adenauer Foundation, Germany (Promotionsförderung der Konrad-Adenauer-Stiftung) during her PhD.

ACKNOWLEDGMENTS

We thank Christiane Steeg and Ulricke Richardt for their excellent technical assistance. We thank Johannes Brandi for his excellent support for best practices in spectral flow cytometry with Cytek Aurora.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.866179/full#supplementary-material

Supplementary Figure 1 | Representative gating strategy for T cell analysis (spleen) acute phase and longitudinal analysis. **(A)** Representative strategy to define effector $CD3^+CD8^+$ T cells based on the expression of CD44 and CD62L. Co-inhibitory receptors were gated within $CD44^+CD62L^-$ $CD8^+$ T cell population (highlighted in blue). Representative gating strategy of **(B)** TIM-3, **(C)** PD-1, **(D)** LAG-3 and **(E)** TSKB20 induced by the infection with *T. cruzi* 15 and 30 dpi. Longitudinal analysis of co-inhibitory receptors in $CD44^+CD62L^-$ $CD8^+$ T cells from spleen and sk. muscle for **(F)** TIM-3, **(G)** PD-1 and **(H)** LAG-3.

Supplementary Figure 2 | Representative gating strategy for T cell analysis in the muscle in the acute phase. Representative strategy to define effector $CD3^+$ $CD8^+$ T cells based on $CD44^+$ and $CD62L^-$expression. Co-inhibitory receptors were gated within $CD44^+CD62L^-$ $CD8^+$ T cell population. Representative gating strategy of **(B)** TIM-3, **(C)** GrB and **(D)** CD107a induced by the infection with *T. cruzi* at 15 and 30 dpi. Absolute numbers of infiltrating lymphocytes in muscle per gram tissue at 15 dpi **(E)** $CD3^+$, **(F)** $CD8^+$, **(G)** $TSKB20^+$. **(H)** Percentage of TIM-3^+ and PD-1^+ out of $TSKB20^+$ effector $CD8^+$ T cells from muscle at 15 dpi. **(I)** Boolean gating analysis of TIM-3, GrB and CD107a. The box plots show the median and range of each combination (15 dpi is depicted in black and 30 dpi in blue). Data from two experiments were compared using the Kruskal-Wallis test and Dunn's Multiple Comparison Test.

Supplementary Figure 3 | Representative gating strategy for T cell analysis in muscle during the chronic phase. Representative strategy to define effector $CD3^+$ $CD8^+$ T cells based on CD44 and CD62L expression. Co-inhibitory receptors were gated within $CD44^+CD62L^-$ $CD8^+$ T cell population (highlighted in blue). Representative gating strategy of **(A)** not infected and **(B)** *T. cruzi*-infected mice 800dpi.

Supplementary Figure 4 | Analysis of co-inhibitory receptors on effector $CD8^+$ T cells from muscle tissue. Upper line: Flow cytometric analysis of T_{eff} from muscle 800 dpi. Results are shown as percentages. **(A)** T_{eff} cells out of $CD8^+$ T cells, **(B)** TIM-3, **(C)** LAG-3 and **(D)** GrB gated within $CD8^+$ T_{eff}. Bottom line: T cells were isolated from the spleen of infected mice at 800 dpi and analyzed by flow cytometry. Results are expressed as absolute numbers per organ. **(E)** $CD8^+$ T cells. **(F)** Comparison of percentage of $CD69^+$ cells in $CD8^+$ T_{eff} cell population from spleen and muscle of infected mice. **(G)** Pearson correlation between absolute numbers of $CD8^+$ T_{eff} cells from muscle per gram and absolute $CD8^+$ T_{eff} cells from spleen (organ). Data from two independent experiments are presented as mean. Results were analyzed for statistical differences with a two-tailored Mann-Whitney test (n.i. n=5; Infected n=20).

Supplementary Figure 5 | Differential PD-1 expression between immunodominant *trans*-sialidase-specific and bulk *T. cruzi*-specific $CD8^+$ T cells. $CD8^+$ T_{eff} from muscle tissue of *T. cruzi*-infected mice 800 dpi were stained using an MHC-I dextramer against the immunodominant TSKB20 epitope and co-stained

with anti-PD-1. **(A)** Bulk $CD44^+CD62L^-CD8^+$ T_{eff} and *T. cruzi*-specific $TSKB20^+$ $CD8^+$ T_{eff} from the muscle of *T. cruzi*-infected mice at 800 dpi were surface stained with anti-PD-1. **(B)** TOX was intracellularly stained in bulk $CD44^+CD62L^-CD8^+$ T_{eff} cells isolated from the muscle of *T. cruzi* infected mice and is positively correlated with the expression of PD-1.

Supplementary Figure 6 | Stimulation of T cells derived from muscle tissue of infected CD45.2 mice with *T. cruzi* lysate-pulsed splenocytes from CD45.1 mice as APC. **(A)** Gating strategy of not infected splenocytes from CD45.1 mice used as antigen-presenting cells (highlighted in orange). **(B)** PD-1 and TOX gating on infected cells in $CD45.1^+$ cells and their capability to produce IFN-γ. **(C)** Gating strategy for infected muscle cells from CD45.2 mice, defined as "effector cells" (highlighted in blue). **(D)** Representative plot of the PD-1 and TOX co-expression gated on $CD44^+CD8^+$ T cells. **(E)** Comparison of the capability to produce IFN-γ, TNF-α and GrB between $PD\text{-}1^+TOX^+$ and $PD\text{-}1^-TOX^-$ T cells. **(F)** FMO controls were used to set the gates for CD44, PD-1 and TOX.

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frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fimmu.2022.866120

Role of the PD-1/PD-L1 Pathway in Experimental *Trypanosoma cruzi* Infection and Potential Therapeutic Options

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Chagas disease (CD) is a neglected chronic infection caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). A significant portion of infected people develops cardiac or digestive alterations over a lifetime. Since several chronic infections associated with antigen persistence and inflammation have been shown to lead to T cell exhaustion, new therapies targeting co-inhibitory receptors to regain T cell activity are under consideration. This study explored immune therapeutic approaches targeting the inhibitory PD-1/PD-L pathway in an experimental model for CD. Infected PD-L1 knockout mice (PD-L1 KO) showed increased systemic parasitemia in blood although no significant differences in parasite load were observed in different organs. Furthermore, we found no significant differences in the frequency of activated T cells or proinflammatory cytokine production when compared to WT counterparts. PD-L1 deficiency led to the production of IL-10 by $CD8^+$ T cells and an upregulation of Tim-3 and CD244 (2B4). Unexpectedly, the lack of PD-L1 did not contribute to a significantly improved T cell response to infection. Single blockade and combined blockade of PD-1 and Tim-3 using monoclonal antibodies confirmed the results observed in infected. PD-L1 KO mice. Our results describe for the first time that the interruption of the PD-1/PD-L1 axis during acute *T. cruzi* infection does not necessarily enhance the immune response against this parasite. Its interruption favors increased levels of parasitemia and sustained upregulation of other co-inhibitory receptors as well as the production of regulatory cytokines. These results suggest that the clinical application of immune therapeutic approaches targeting the *PD-1/PD-L1* axis in CD might be risky and associated with adverse events. It highlights that more research is urgently needed to better understand the immune regulation of T cells in CD before designing immune therapeutic approaches for a clinical context.

Keywords: *Trypanosoma cruzi*, co-inhibitory receptors, Chagas Disease, PD-L1, PD-1, Tim-3.

OPEN ACCESS

Edited by:
Anton Götz,
National Institutes of Health (NIH), United States

Reviewed by:
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Carolina Verónica Poncini,
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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *30 January 2022*
Accepted: *23 May 2022*
Published: *23 June 2022*

Citation:
Arana Y, Gálvez RI and Jacobs T (2022) Role of the PD-1/PD-L1 Pathway in Experimental Trypanosoma cruzi Infection and Potential Therapeutic Options. Front. Immunol. 13:866120. doi: 10.3389/fimmu.2022.866120

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) is the etiological agent of Chagas disease (CD). This chronic disabling disease is endemic in 21 Latin American (LA) countries, where approx. 6 million people are estimated to be infected and 70 million live at risk of contracting infection according to the WHO (1). Migratory movements from LA have spread this disease to all continents

and it has become a serious health problem in countries like the US and Spain. There is no vaccine licensed, and the treatments accessible today exhibit severe side effects (2, 3). The natural mechanism of transmission is the direct contact with the contaminated feces released from the vector, a hematophagous triatomine bug, after a blood meal. The infective trypomastigotes invade the host skin or mucosa starting the acute phase, a period of intracellular proliferation and systemic dissemination accompanied by increased parasite numbers in blood and tissues. Although intense inflammatory reactions and immune activation occur, patients might not necessarily show defined clinical symptoms (4, 5). This in consequence leads ultimately to an underdiagnosis of this infection. The adaptive immune response controls the acute infection in most cases but fails to eliminate the parasites leading to the establishment of asymptomatic chronic infection. Several years later, about 30 % of the infected individuals become symptomatic developing pathologies associated with cardiac or digestive tissues, as well as mixed forms, which untreated can lead to death (6). It has been demonstrated that $CD4^+$ and $CD8^+$ T cells play a crucial role in the control of acute and chronic *T. cruzi* infection (5, 7, 8). Furthermore, *T. cruzi* has developed several strategies to evade immune responses. It has been demonstrated that during the acute phase, lymphocyte activation is suppressed by the production of immune-modulatory molecules (i.e. GPI-anchored mucins, *trans*-sialidases) (9), as well as the inhibition of IL-2R expression (10) promoting immunosuppression that contributes to the spread of the infection. Recently the concept of T cell exhaustion has arisen and been studied in several models of chronic viral infection (11, 12) and several studies have extended this concept to infectious diseases due to intracellular protozoa such as *Toxoplasma gondii*, *Leishmania major*, *Plasmodium* spp., and *T. cruzi* (13–19). Therefore we asked if the increased expression of co-inhibitory receptors could be an additional escape mechanism during acute *T. cruzi* infection. Several studies have demonstrated that co-inhibitory receptors, especially the *PD-1/PD-L1* pathway plays a central role in regulating T cell exhaustion. Its blockade reinvigorates exhausted $CD8^+$ T cells, leading to a reduced pathogen burden (11, 20). However, the impact of the PD-1/PD-L1 pathway in acute *T. cruzi* infection is still controversial. Previous studies have shown that *T. cruzi* can modulate the expression levels of co-inhibitory receptors such as PD-1 during experimental infection (5, 8, 21). However, many of these observations were collected from *ex vivo* experiments where the infection models employed different parasite and mice strains with conflicting results. Here, we evaluated the role of the PD-1/PD-L inhibitory pathway during infection with the *T. cruzi* Tulahuen strain to unveil potential intervention points and therapeutic strategies to increase parasite clearance and avoid a progression to the chronic phase. The T cell response was evaluated in PD-L1 KO mice and subsequently, a single blockade and a combined blockade of PD-1 and TIM-3 using monoclonal antibodies were applied as a potential therapeutic intervention in WT mice. We demonstrate that the interruption of the PD-1/PD-L pathway neither reduces parasitemia nor improves the outcome of *T. cruzi* infection. Contrary to our expectations, its interruption favors a higher parasitemia and a pronounced induction of other co-inhibitory receptors like Tim-3 and CD244. Additionally, it induces the secretion of the anti-inflammatory cytokine IL-10.

In conclusion, our data provide evidence that despite the upregulation of PD-1 and its receptor PD-L1, this immune regulatory pathway does not limit the protective immune response against *T. cruzi* infection.

MATERIALS AND METHODS

Mice

7-8 weeks old C57BL/6J (WT) and PD-L1KO on the C57BL/6J background mice were bred under specific pathogen-free conditions at the BSL-3 animal facility at Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg. Mice were infected with *T. cruzi* by intraperitoneal (i.p.) inoculation of 2 x 10^3 bloodstream trypomastigotes diluted in 200 µL of DPBS (PAN-BIOTECH), obtained from infected passage mice. Control mice received 200 µL of DPBS alone. To monitor parasitemia during infection, 2 µL of blood samples were taken from tail vein puncture at the indicated time points. Parasites were counted using a Neubauer chamber (0.02 mm thickness). Mice were euthanized by CO_2 inhalation and a subsequent neck dislocation.

Parasites

In vivo passage of *T. cruzi* Tulahuen strain was achieved by i.p. inoculation of mice with 5 x 10^5 bloodstream trypomastigotes resuspended in 200 µL of DPBS. Periodic passages took place every 15 days. For *in vitro* experiments, cell culture-derived *T. cruzi* trypomastigotes were obtained from the supernatant of infected 86Hg39 cells (BNITM) maintained in complete RPMI 1640 medium (PAN-BIOTECH) supplemented with 10 % of fetal calf serum (PAN-BIOTECH), 1 % L-Glutamine (PAN-BIOTECH), and 0.5 %, Gentamycin sulfate (PAA) at 37 °C and 95 % CO_2 after 3-4 days post-infection.

Generation of Bone-Marrow-Derived Dendritic Cells

Hematopoietic stem cells from the bone marrow were isolated under sterile conditions and 3 x 10^6 cells were seeded with a complete BMDCs medium composed of DMEM, 10 % fetal calf serum, 1 % L-Glutamine, 0.5 %, Gentamycin, and 10 % GM-CSF. On days three and six after bone marrow isolation, 8 mL of complete BMDCs medium were added additionally to the culture. On day seven, BMDCs were fully differentiated and used for further experiments. For PD-L1 expression analysis, BMDCs were co-incubated in a ratio of 1:1 *T. cruzi* trypomastigote per cell for 6 hours. Afterward, cells were washed twice, resuspended in a complete BMDCs medium, and incubated for 72 hours. Next, the BMDCs were stained with fluorescent antibodies against surface markers before fixation. BMDCs were then permeabilized (eBioscience Foxp3/Transcription Factor Staining Buffer Set) and stained for intracellular *T. cruzi* with anti-*T. cruzi* polyclonal rabbit

antiserum in a 1:200 dilution (BNITM). PD-L1 expression was evaluated by flow cytometry and BMDCs isolated from PD-L1 KO mice were included as an internal control of PD-L1 expression.

Organ Sampling

Mice were sacrificed at different time points post-infection as indicated in the figures. To analyze parasitic load, 25 mg tissue samples of heart, liver, skeletal muscle and 10 mg spleen were harvested, and rinsed in DPBS, to avoid contamination with blood parasites. Samples were stored in liquid nitrogen until DNA isolation. For flow cytometry, the spleen was harvested and lymphocytes were isolated. Briefly, the spleens were collected at 4°C in RPMI, mashed through a 70-µm-pore-size cell strainer, and centrifuged at 315 g for 5 min at 4°C. After that, RBC lysis was done for 5 min at RT. Subsequently, the cells were centrifuged and washed again, then resuspended in 10 mL sterile complete RPMI 1640 medium and passed through a 40-µm-pore-size cell filter. Finally, the cell number was determined.

Flow Cytometry Analysis

3 x 10^6 cells were used for surface and intracellular staining. Briefly, splenocytes were incubated with the antibody cocktail diluted in Fc-block at 4 °C and for 30 min in the dark. After surface antibody incubation cells were washed. For intracellular staining, cells were stimulated with 50 ng/mL PMA (SIGMA) and 500 ng/mL Ionomycin (SIGMA) for six hours at 37°C and 5 % CO_2, and 2 µM Monensin (BioLegend) was added during the last 5 hours of culture. Cells were washed twice with cold DPBS (315 g, 5 min, and 4°C), and stained with antibodies directed against surface antigens (as described above). In the next step, the cells were fixed and permeabilized according to the manufacturer's protocol of the Foxp3/Transcription factor staining buffer set. Briefly, after surface staining, cells were washed twice with cold DPBS and fixed with 100 µL fixation buffer for 30 min at RT. After that cells were washed twice with permeabilization buffer and stained with fluorescently labeled anti-cytokine antibodies diluted in permeabilization buffer for 30 min at RT. After incubation, cells were washed twice with permeabilization buffer and suspended in 200 µL of FACS buffer (1 % FCS, 0.1 % sodium azide in PBS) for fluorescence measurements on a BD LSRII flow cytometer (BD, Biosciences, Heidelberg). FMO controls were used for gating. A complete list of antibodies used is given as **Supplementary Material** in **Table 1**. Cytometry data was analyzed using FlowJo 10.8.1.

Cytometric Beads Assay-LEGENDplex™

Cytokine profile was determined using the LEGENDplex™ Mouse Th Cytokine Panel (13-plex). Serum samples were processed following the manufacturer's instructions, afterwards, the samples were measured with the Accuri C6 cytometer (Accuri Cytometer Inc., Ann Arbor).

In Vivo Blocking Assays

For PD-1 blockade, 0.2 mg of anti-PD-1 mAb (RMP1-14, BioLegend) was administered i.p. at the time of infection and a second dose, 7 days post-infection. Control mice were administered with the same amount of rat IgG2a isotype control (RTK2758, BioLegend). For blockade of PD-1 and TIM-3, 0.2 mg of anti-PD-1 mAb and 0.2 mg of anti-TIM-3 (RMT3-23, BioLegend) were administered following the same scheme as applied for the PD-1 blockade. The control group received 0.2 mg of rat IgG2a isotype control.

Parasite Detection With Quantitative Real-Time PCR

Frozen tissue samples were mechanically disrupted and homogenized. The tissue suspension was incubated overnight at 56 °C with lysis buffer and Proteinase K from QIAamp DNA Mini Kit (QIAGEN) and DNA isolation was performed according to manufacturer specifications. The concentration of DNA was determined using a NanoDrop 2000 Spectrophotometer (PeqLab/Thermo Scientific). The standards for the qPCR were generated by spiking tissue homogenates from naive mice to which 10^5 cell-cultured *T. cruzi* trypomastigotes were added. DNA was isolated as mentioned above and serially diluted with 25 µg/mL DNA isolated from unspiked naive mice tissue. The 10-fold dilution series contained DNA from 10^5 to 10^{-2} parasites, equivalents per 50 ng of total DNA. A standard curve was generated from these standards, in triplicate reactions, to determine the parasitic load in the organs of infected mice. Real-time PCR Mastermix was prepared using the QuantiTec SYBR Green PCR Kit (QIAGEN) and run on a Rotor-Gene (R Corbett Research). Primers target the minicircle variable region from kDNA and amplify a 330 bp fragment. The amount of T. cruzi from kinetoplast DNA (kDNA) was quantified in the mouse-GAPDH housekeeping gene. Samples were analyzed by duplicates. Primer sequences were as follows: Tc 121F 5′–AAATAATGTACGGGKGAGATGCATGA-3′, Tc 121 R 5′–GGTTCGATTGGGGTTGGTGTAATATA -3′, GAPDH-F 5′-GTCGGTGTGAACGGATTTGG-3', and GAPDH-R 5'-TTCCCATTCTCGGCCTTGAC-3'. Thermal Profile: Initial $_{DNA\ denaturation}$ = 95°C, 900 sec; ($T_{DNA\ denaturation}$ = 94°C, 60 sec; $T_{primer\ annealing}$ = 68°C, 60 sec; $T_{elongation}$ = 72°C, 60 sec) x 5; following by ($T_{DNA\ denaturation}$ = 94°C, 45 sec; $T_{primer\ annealing}$ = 64°C, 45 sec; $T_{elongation}$ = 72°C, 45 sec) x 40; Final $_{elongation}$ = 72°C, 600 sec. A melting curve phase program was applied with the continuous measurement between 62°C and 95°C. Duplicate values for each DNA sample were averaged (geometric mean) and parasite equivalent load was calculated automatically using the Rotor-Gene 6000 Series Software 1.7 (Corbett research/Qiagen) Briefly, we plotted the Ct value against each standard of known concentration and calculated the linear regression line of this curve. To normalize the amount of DNA, GAPDH was used to correct the initial sample mount. Murine GADPH and Tc121/122 amplification have the same efficiency. The parasite loads below the limit of quantification (LOQ), which means less than 1 parasite equivalent, were set to LOQ/2 (0.05 parasite equivalents per 50 ng of DNA), as previously described in (22, 23).

Statistical Analysis

Data were analyzed for normal distribution before running statistics. Statistical analyses were performed in GraphPad Prism 9.3.0. If not otherwise stated in the legend, the Kruskal-Wallis test was used to determine statistical differences followed by a pairwise comparisons analysis using Dunn's test. Non-significant results

($p>0.05$) were not mentioned or plotted to avoid busy figures. All data are shown as mean ± SEM.

RESULTS

T. cruzi Infection Induces Expression of PD-L1 on Antigen-Presenting Cells

To test whether *T. cruzi* infection induces expression of the PD-1 ligand PD-L1 on antigen-presenting cells (APCs), BMDCs from WT mice were infected *in vitro* with *T. cruzi* trypomastigotes and PD-L1 expression was evaluated by flow cytometry. The experimental setup, gating strategy, and representative dot plots are depicted in **Figures S1A, B**. CD11c and CD86 expression were employed to define BMDCs and infected cells were identified by staining with an anti-*T. cruzi* polyclonal rabbit antiserum. Results showed a clear upregulation of PD-L1 on BMDCs infected with *T. cruzi* compared to non-infected cells during *in vitro* infection (**Figure 1A**). Specific induction of PD-L1 after *T. cruzi* infection *in vivo* was measured at 22-24 days post-infection (dpi). The gating strategy and representative dot plots are depicted in **Figure S2A**. For this analysis, spleens were harvested and antigen-presenting cells were characterized by flow cytometry. Dendritic cells (DCs) were defined as $CD3^-CD11b^-CD11c^+$ while classical macrophages (MΦ) were defined as $CD3^-CD11c^-C11b^+Ly6C^+$. Infected WT mice showed an 8-fold higher frequency of PD-$L1^+$ DCs than those from non-infected (n.i.) WT mice (**Figure 1B**). WT mice had a significantly higher frequency of PD-$L1^+$MΦ after infection, in mean 37% PD-$L1^+$MΦ were found, which is 15-fold more compared to n.i. WT counterparts (**Figure 1C**). As expected DCs and MΦ from PD-L1 KO mice did not express PD-L1. We also analyzed the expression of PD-L2 to prove the possibility of a compensatory role of this ligand. Our results revealed a low frequency of PD-$L2^+$ DCs in n.i. WT and PD-L1 KO mice. The infection led by trend to a small increase of PD-$L2^+$ DCs in both mice strains which was not statistically significant (**Figure S2B**). However, the PD-L2 expression increased statistically significantly on $CD11b^+$ $Ly6C^+$ $Ly6G^-$ MΦ from WT mice at 22-24 dpi (**Figure S2C**). These data show that in our model infection with the *T. cruzi* Tulahuen strain can induce PD-L1 expression on host cells.

Co-inhibitory Receptors PD-1, Tim-3, and CD244 Are Expressed on T Cells During Acute *T. cruzi* Infection *in vivo* and PD-L1 Deficiency Reinforces Their Expression

We next investigated if *T. cruzi* infection modulates the expression of co-inhibitory receptors *in vivo*. WT mice were infected and sacrificed at two different time points: 10 to 15 dpi and 22 to 24 dpi. Spleens were harvested, and $CD4^+$ and $CD8^+$ T cells were further characterized by flow cytometry. Representative plots and the gating strategy are depicted in **Figures S3A, B**. Infected WT mice displayed significantly higher frequencies of PD-1^+ $CD4^+$ T cells at the beginning of the acute phase (10 -15 dpi) compared to n.i. mice and also compared to PD-L1KO mice. We observed that

PD-1 expression in the WT mice was only transient since at the peak of parasitemia at 22-24 dpi the PD-1 expression had already decreased to levels comparable to n.i. mice, while their PD-L1 KO counterparts strongly upregulated the expression of

PD-1 (51.8 % of all $CD4^+$ T cells) towards the end of the acute stage (**Figure 2A**). The upregulation of PD-1 on $CD8^+$ T cells was not as dynamic as on $CD4^+$ T cells, since PD-1 expression at 10-15 dpi did neither increase in WT mice nor PD-L1 KO mice. In WT mice the PD-1 expression also did not increase at the peak of parasitemia. Only at 22-24 dpi, the frequency of PD-1^+CD8^+ T cells increased in PD-L1KO mice (**Figure 2B**). These results demonstrate that *T. cruzi* infection induces PD-1 on $CD4^+$ T cells from WT and PD-L1 KO mice. $CD8^+$ T cells neither in WT nor in PD-L1 KO mice respond to infection with the upregulation of PD-1. Only in infected PD-L1 KO mice did the expression of PD-1 on $CD8^+$ T cells strongly increased. Next, we evaluated, if the expression of Tim-3 was induced after *T. cruzi* infection and if it was affected by the interruption of PD-1/PD-L1 signaling in PD-L1 KO mice. The results in **Figure 2C** for $CD4^+$ T cells and **Figure 2D** for $CD8^+$ T cells showed no significant increase in

Tim-3 expression in WT or PD-L1KO mice in comparison to n.i. mice at 10-15 dpi. However, on 22-24 dpi Tim-3^+CD4^+ T cells increased in WT and PD-L1KO mice to a comparable extent (13.09 % and 14.93 % respectively) but the increase of

Tim-3^+ $CD8^+$ T cells was more pronounced, reaching 25.40 % in WT mice and 36.33 % in PD-L1 KO mice. These results confirmed that in this acute infection model, Tim-3 might represent a compensatory regulatory mechanism for the disrupted

PD-1/PD-L1 pathway, mainly affecting $CD8^+$ T cells. Finally, we aimed to evaluate the expression of CD244 (2B4). CD244 is found on many immune cells but mainly on NK cells and activated $CD8^+$ T cells. The contribution to the effector function of T cells needs further investigation since it is not completely clarified if it acts co-stimulatory or co-inhibitory (20, 24). In the context of parasitic diseases, it has been barely explored. Since only one report analyses CD244 function and shows an upregulation in the chronic human CD (25), we wanted to elucidate if CD244 represents a potential regulatory pathway in the acute stage of infection. In **Figure 2E** the expression of CD244 on $CD4^+$ and in **Figure 2F** on $CD8^+$ T cells is depicted. The kinetic of CD244 expression followed a similar pattern as the Tim-3 expression, meaning that at

10-15 dpi neither WT nor PD-L1 KO mice upregulated this receptor in comparison to n.i. mice. The expression of CD244 increased significantly on $CD4^+$ T cells in WT and PD-L1 KO mice at 22-24 dpi. The induction of CD244 was also found on $CD8^+$ T cells at 22-24 dpi in WT mice and even stronger and highly statistically significant in PD-L1 KO mice. These results support the idea of a compensatory pathway *via* CD244 after disruption of PD-1/PD-L1 signaling.

PD-L1 Deficiency Does Not Affect Activation, IFN-γ Production, or Granzyme B Production but Induces IL-10 Secretion by $CD8^+$ T Cells

To evaluate an early effect of PD-L1 deficiency in the control of acute *T. cruzi* infection by $CD4^+$ and $CD8^+$ T cells, splenocytes from WT mice and PD-L1 KO mice were isolated and stimulated

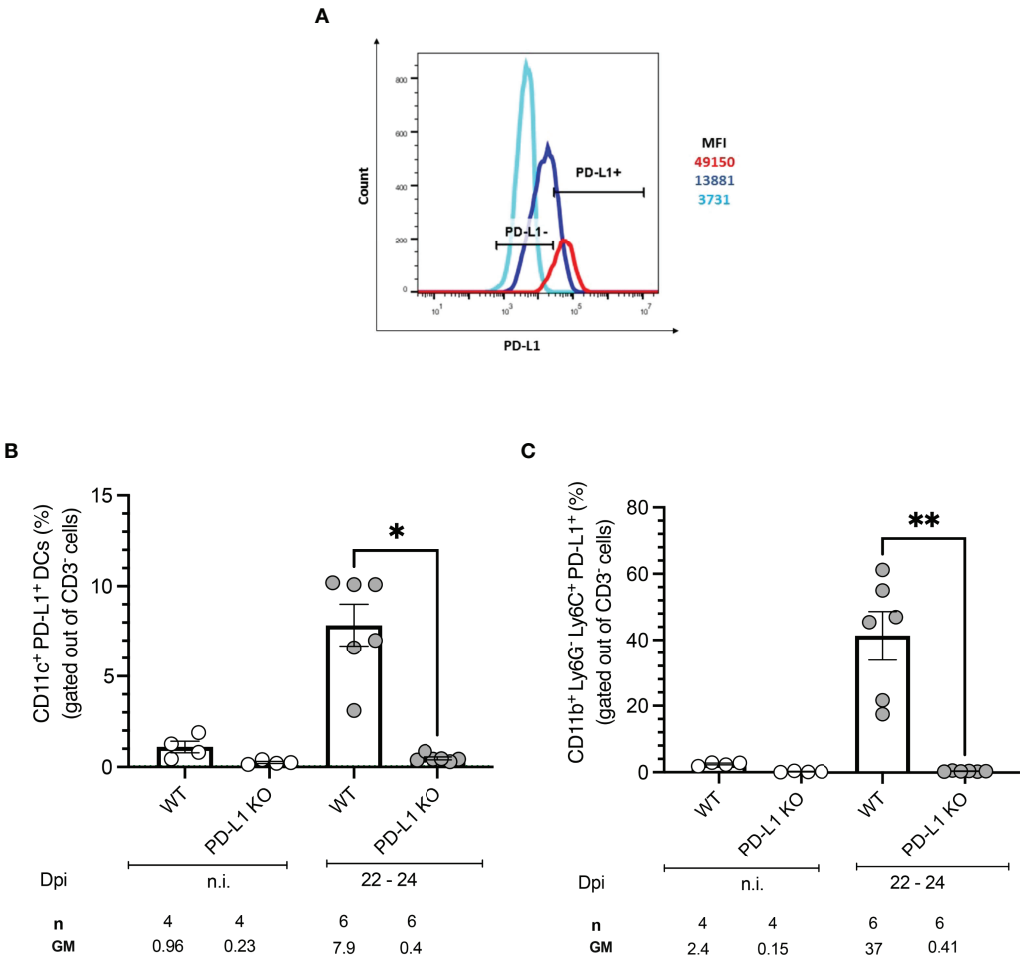

FIGURE 1 | PD-L1 expression on antigen-presenting cells after *in vitro* and *in vivo* infection with *T. cruzi*. BMDCs were infected with *T. cruzi in vitro*. After 72 h the PD-L1 Expression was analyzed by flow cytometry. **(A)** The Histogram shows the MFI of PD-L1 expression on infected (red) and non-infected (blue) BMDCs. FMO staining control (light blue) was used to identify and gate the PD-L1cells population. Expression of PD-L1 on APCs from WT and PD-L1 KO mice infected with *T. cruzi*. On day 22- 24 post-infection spleens from infected and n.i. mice were isolated and the expression of PD-L1 was evaluated by flow cytometry. **(B)** Expression of PD-L1 on $CD11b^-$ $CD11c^+$ (DCs). **(C)** Expression of PD-L1 on $CD11b^+$ $Ly6G^-Ly6C^+$ MΦ. Data shown are corresponding to two independent experiments n.i. = 4; infected =6. Asterisks denote P values of <0.05. *P<0.05 and **P<0.01.

with PMA/Ionomycin. Functionality was measured by analysis of IFN-γ and IL-10 production. Cytotoxicity was evaluated based on Granzyme B production. PD-L1 deficiency did not induce significant differences in the frequency of activated $CD4^+$ and $CD8^+$ T cells in comparison to infected WT mice (**Figures 3A, B**). The *T. cruzi* infection led to a strong increase in IFN-γ production by $CD4^+$ and $CD8^+$ T cells. The capability to produce IFN-γ was not affected by PD-L1 deficiency (**Figures 3C, D**). The results in **Figure 3E** show that PD-L1 deficiency is accompanied by increased production of IL-10 by $CD8^+$ T cells at 22-24 dpi in comparison to infected WT mice. Granzyme B expression only slightly increased at 10-15 dpi in both WT and PD-L1 KO mice without reaching statistical significance. At 22-24 dpi only WT mice showed a significant increase of Granzyme B in comparison to n.i. counterparts (**Figure 3F**).

No Effect of Combined Therapy by Monoclonal Antibodies Against PD-1 and Tim-3 During *T. cruzi* Infection *In Vivo*

Due to the upregulation of PD-1 and Tim-3 on $CD4^+$ and $CD8^+$ T cells after *T. cruzi* infection and due to their increased induction in PD-L1 KO mice, a combined blockade of PD-1 and Tim-3 was assumed to be a potential therapeutic intervention. On the one hand to increase the functionality of $CD4^+$ T cells, unleash cytotoxic mechanisms of $CD8^+$ T cells, and thus promote better parasite elimination. On the other hand, it potentially blocks compensatory mechanisms between both pathways. **Figure 4A** shows the experimental setup. WT mice were infected with *T. cruzi* and treated with monoclonal antibodies against PD-1 and Tim-3 or with an isotype control antibody. The results depicted in **Figure 4B** showed that the

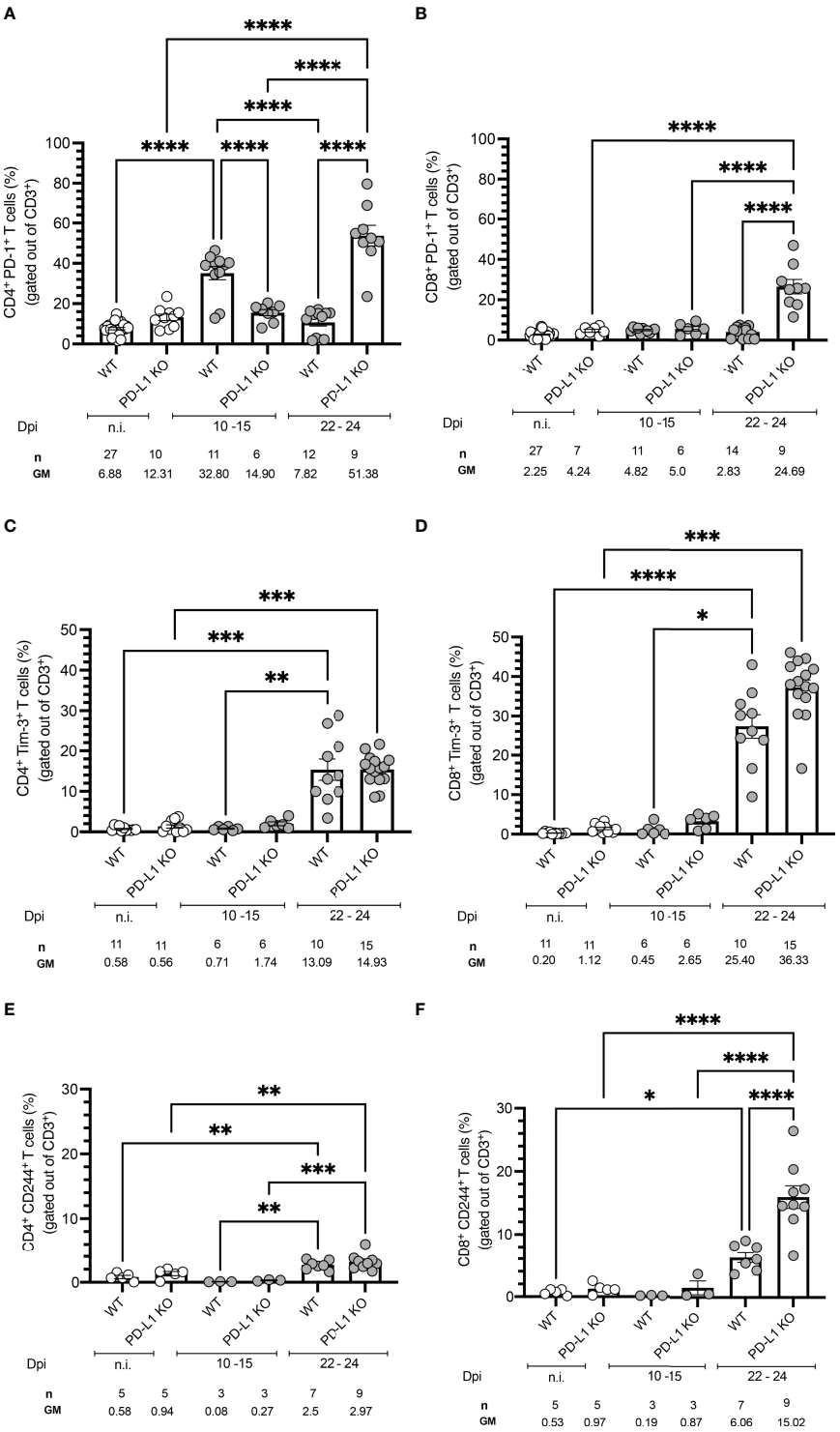

FIGURE 2 | Time course of co-inhibitory receptors expression on T cells from WT and PD-1 KO mice infected with *T. cruzi*. Time-course experiments showing expression of **(A)** PD-1 on $CD4^+$ and **(B)** $CD8^+$ T cells; **(C)** Tim-3 on $CD4^+$ and **(D)** $CD8^+$ T cells; **(E)** CD244 on $CD4^+$ and **(F)** $CD8^+$ T cells. Error bars indicate standard errors of the means (SEM). Data from three independent experiments. Under the graphs, n is the absolute number of mice used per time point, and the values below are the geometric means (GM). Data were analyzed for statistical significance using the Kruskal-Wallis test following Dunn´s multiple comparisons test. Asterisks denote P values as described in Methods; ns (not significant) was not plotted to avoid a busy figure.

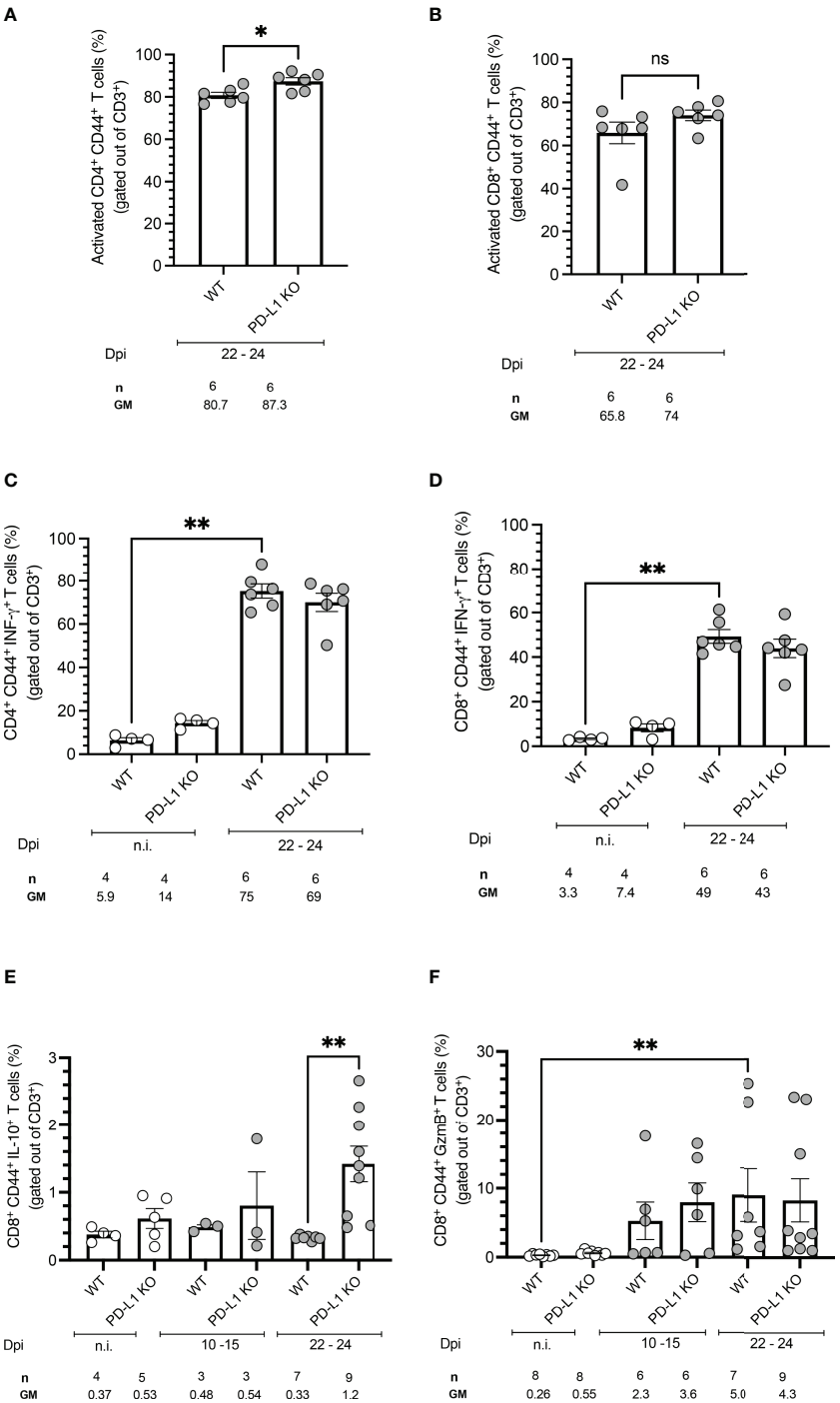

FIGURE 3 | PD-L1 deficiency does not affect activation, IFN-γ production, or Granzyme B production but induces IL-10 secretion on $CD8^+$ T cells after stimulation. Spleen cells were isolated from mice and analyzed by flow cytometry after a 5 h stimulation with PMA/Ionomycin. **(A, B)** PD-L1 deficiency does not affect T cell activation, defined by CD44 expression, after *T. cruzi* infection on 22-24 dpi. **(C, D)** *T. cruzi* infection induces IFN-γ on $CD4^+$ and $CD8^+$ T cells from WT and PD-L1 Ko mice. PD-L1 deficiency does not affect IFN-γ production. **(E)** IL-10 is only induced at 22-24 dpi in $CD8^+$ T cells from PD-L1KO Mice **(F)** Granzyme B is significantly upregulated only on $CD8^+$ T cells from WT mice. PD-L1 deficiency does not affect Granzyme B production. Error bars indicate standard errors of the means (SEM). Data from two independent experiments. Under the graphs, n is the absolute number of mice used per time point, and the values below are the geometric means (GM). Data were analyzed for statistical significance using the Kruskal-Wallis test following Dunn´s multiple comparisons test. Asterisks denote P values as described in Methods; ns (not significant) was not plotted to avoid busy figures.

combined blockade had no impact on the control of the parasitemia in blood. Also, the resistance to the infection was not improved, since no significant differences were observed regarding the bodyweight (**Figure 4C**). To evaluate the effect on the systemic inflammatory response, serum samples were collected and pooled and their cytokine profile was evaluated by a cytometric beads assay. After the combined blockade IL-10 could be detected in the serum of infected mice while it was not detectable in the pooled sera from infected mice treated with the isotype control antibody, confirming the results seen in the infection of PD-L1 KO mice (**Figure 4D**). Furthermore, TNF-α was present in sera from both groups in a comparable concentration, suggesting not to be affected by the combined blockade (**Figure 4E**). Using this experimental approach, it was possible to detect IFN-γ in pooled sera from infected mice (200 pg/mL) and the combined blockade induced a notable increase of systemic IFN-γ (> 400 pg/mL) (**Figure 4F**). Finally, we evaluated the effect of the combined blockade on the tissue parasite load during *T. cruzi* infection. For this, tissue samples from the spleen, liver, heart, and skeletal muscle corresponding to the infected mice treated with blocking antibodies and their respective isotype controls were isolated and the parasite load was evaluated by qRT-PCR. The results shown in **Figure 4G** do not exhibit significant differences in the parasite load in the analyzed tissues between infected mice that received a combined blockade and their respective isotype control-treated mice. However, by trend, an increased parasite load was observed in the group that received the combined blockade. Parasitemia in skeletal muscle was significantly higher than the parasitemia found in the liver tissue.

DISCUSSION

After acute infection with *T. cruzi*, which is associated with a high antigen load and production of pro-inflammatory cytokines, antigen-specific T cells, especially $CD8^+$ T cells, reduce the pathogen load. However, the immune response often fails to clear residual pathogen reservoirs in a specific tissue leading to chronic infection. Chronic antigen exposure is accompanied by a gradual loss of T cell effector function leading ultimately to T cell exhaustion (11, 12). In various settings, it was already shown that exhausted T cells expressing PD-1 can be revigorated, at least in part, by blocking the PD-1/PD-L1 pathway (20). Therefore, manipulation of this pathway might pave new avenues for the treatment of parasitic infections some of which are known to persist lifelong.

The importance of the PD-1/PD-L inhibitory pathway has been studied previously in the context of *T. cruzi* induced acute myocarditis (5) and a chronic model of Chagas heart disease in mice (8). Whereas the first publication reported an increased cardiac inflammatory response, reduced blood parasitemia, and cardiac parasitism, along with high mortality rates upon PD1/PD-L blockade; the second showed that

PD-1/PD-L1 blockade together with an immunization using irradiated *T. cruzi* only decreased the blood parasitemia but did not affect cardiac parasite load nor cytokine production in the heart tissue. These conflicting results reflect the major problem in CD research. There are up to date no standardized animal models to study *T. cruzi* infection and subsequent CD. Using different isolates and strains of *T. cruzi* parasites introduce a serious bias and differences in the immune response from mice with different genetical background leads to potential different outcomes. Moreover, there is neither a consensus about standardized routes of infection (intra-peritoneal, intravenous, subcutaneous, oral, congenital) nor on the assessment of parameters to characterize the acute and chronic infection or their pathological consequences in the different infected tissues (26–29). Therefore, it is essential to highlight that our study was focused on a model established on the intraperitoneal infection of female C57BL/6 mice with *T. cruzi* trypomastigotes of the reticulotropic *T. cruzi* strain Tulahuen (DTU IIe) (30). Our results are limited to the acute stage of infection until day 28 post-infection since the majority of PD-L1 KO mice succumb to infection after this time point. Trypanosomes have developed several strategies to down-regulate T cell responses, the modulation of the

PD-1/PDL pathway being one of them, that could avoid their complete clearance and allow their persistence (15, 16). Based on this knowledge, we first aimed to address, if *T. cruzi* infection modulates PD-1 expression at the beginning of the infection and if PD-1 expression is necessarily associated with exhaustion of T cells in the acute stage. Second, we aimed to interrupt the PD-1/PD-L1 pathway during the infection using PD-L1 KO mice and subsequently test the therapeutic potential of a therapeutic blockade of PD-1. We demonstrated that PD-L1 expression is upregulated in bone marrow-dendritic cells (BMDCs) after *in vitro* and *in vivo* infection with *T. cruzi*. This confirms previous reports, that used other *T. cruzi* strains (5, 8, 21). This suggests that the induction of PD-L1 is not parasite strain or host-dependent, and emphasizes the involvement of this inhibitory pathway in the context of *T. cruzi* infection. Some common γ -chain (γ c) cytokines (31, 32) and inflammatory cytokines have been described as the driving mechanisms that

up-regulate PD-L1 on APCs or somatic cells in the tissue (32–34). Our results suggest that enhanced levels of PD-L1 observed in infected BMDCs and APCs are associated with the presence of the parasite inside the cells and are not induced by excretory/secretory parasite or cell products since the PD-L1 expression levels on uninfected cells in the same culture remained low and cells incubated with conditioned media from infected cells did not induce PD-L1 (data not shown). These results suggest that *T. cruzi* could induce the PD-L1 expression through activation of other alternative pathways (e.g., signals associated with its intracellular replication). Our results show that infection with *T. cruzi* induces the expression of PD-1 on T cells *in vivo*. Interestingly, we observed statistically significant higher frequencies of $CD4^+$ T cells expressing PD-1 during the course of the infection whereas only a slight increase of $CD8^+$ $PD\text{-}1^+$ T cells was found. Our results support the findings observed during the infection by *T. cruzi* Y, a cardiotropic strain (5), and are in

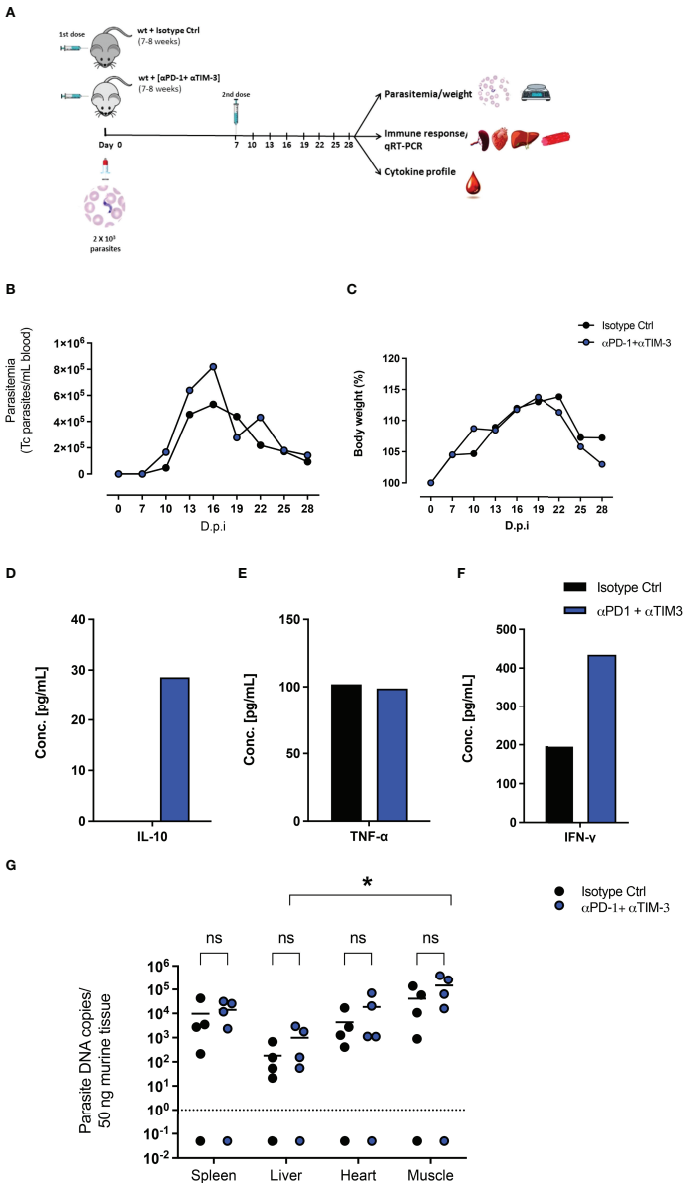

FIGURE 4 | Combined therapeutic intervention with αPD-1 and αTim-3 monoclonal antibodies does not reduce parasitemia but induces IL-10. **(A)** Scheme of infection and combined αPD-1 and αTim-3 therapy. Mice were infected i.p. with 2x10^3 T. cruzi parasites on day 0. They received αPD-1 and αTim-3 in a concentration of 0.2 mg each/dose on day 0 and day 7 after infection. The control group received i.p. 2 doses of isotype antibody in the same concentration. WT mice infected and isotype treated n=8; WT infected and αPD-1+αTIM- 3 n=7. Results from two independent experiments **(B)** Parasitemia and **(C)** body weight were analyzed over the course of infection (28 days) and are shown as means with SD. Whole blood samples were collected, and sera were isolated and pooled. Cytokine levels were determined by a cytometric bead assay. Results are expressed as the cytokine concentration of the pooled sera samples **(D)** IL-10, **(E)** TNF-α, and **(F)** IFN-γ. **(G)** Effect of interruption of PD-1/PD-L1 signaling on parasite load analyzed by *T. cruzi*-specific qRT-PCR. Comparative parasite load in spleen, liver, heart, and skeletal muscle from infected and treated with blocking antibodies against αPD-1+ αTIM-3 or their respective isotype controls. Parasite load was calculated from a standard curve. The standard error of the mean is indicated (SEM). Asterisk denotes P values of < 0.05 by One-way ANOVA compared to isotype control values. P < 0.05*; ns (not significant).

contrast to the common finding that PD-1 is mainly induced on CD8$^+$ T cells during infections with viral pathogens (35). In protozoan parasite infections, CD4$^+$ T cells are critical in the establishment of a Th1 response to activate the microbicidal activity of macrophages and the generation of cytotoxic CD8$^+$ T cells that recognize and kill infected cells (36). The up-regulation of PD-1 on CD4$^+$ T cells might represent an immunoregulatory mechanism to avoid tissue damage caused by an exacerbated inflammatory response (32, 33). The up-regulation of Tim-3 upon blockade of the PD-1/PD-L1 pathway with anti-PD-1

monoclonal antibodies has been associated with the failure of the therapy in the cancer (37, 38). We observed also a significant up-regulation of Tim-3 on $CD8^+$ T cells from infected PD-L1KO mice. Of note, this increase of Tim-3 on $CD8^+$ T cells is also common to other *T. cruzi* strains, since using the *T. cruzi* strain Brazil, described as a myotropic strain that can establish chronic disease, the Tim-3 expression at 22 dpi was also significantly higher compared to WT mice (**Figure S4D**). CD244 (2B4) is a co-inhibitory receptor whose expression is up-regulated on antigen-specific $CD8^+$ T cells in chronic viral infections (20), and it is also up-regulated on $CD8^+$ T cells from chronic Chagas patients with severe forms of the disease (39). Its expression upon PD-L1 deficiency suggests a potential compensatory mechanism of this pathway, that needs to be further analyzed. Interestingly, and in contrast to our expectations, PD-L1 KO mice showed a trend of higher blood parasitemia (**Figure S3B**). This could be due to an impaired parasite clearance and suggests the possibility of a co-stimulatory function for PD-L1. The possibility of a co-stimulatory role for PD-L1 associated with a protective role has been hypothesized for infections with *Listeria monocytogenes* (40) and *Mycobacterium tuberculosis* (41). However, following this hypothesis, it remains striking, that we did not observe a decreased frequency of activated $CD4^+$ $CD44^+$ T cells or $CD8^+$ $CD44^+$ T cells in infected PD-L1 KO mice in comparison to infected WT mice. Interestingly it was shown recently that PD-1 derived signals promote an optimal $CD8^+$ T cell memory formation. Therefore, disruption of the PD-1/PD-l1 pathway may lead to a decreased control of *T. cruzi* at later stages (42).

Since PD-L2 is an alternative ligand of PD-1 and has been reported to compensate for the PD-L1 deficiency in some infections (43), it could be speculated that also in our model PD-L2 might compensate for the missing signaling *via* PD-L1. Its induction during the *T. cruzi* infection has been reported on T cells and macrophages (5, 21) but a compensatory role has yet not been demonstrated. In our exploratory experiments, there was non-significant upregulation of PD-L2 on APC from PD-L1 KO mice in comparison to WT-infected mice (**Figures S2B, C**). An alternative explanation of the impaired parasite clearance could be an activation of other immunoregulatory mechanisms in the absence of PD-1/PD-L1 signaling. The increased expression of IL-10 might represent such a compensatory mechanism. It has been demonstrated that IL-10, a potent regulatory cytokine, is involved in pathogen persistence during chronic infections (36, 44). Previous studies showed that during the infection by intracellular parasites, including *T. cruzi*, IL-10 acts by down-regulating T-cell responses favoring the parasite persistence (7, 36, 45). Moreover, studies in chronic LCMV infection and Toxoplasma showed that PD-L1 and IL-10 are independent pathways and act in parallel to regulate the immune response preventing immune mediated-tissue damage (46–48). There is strong evidence that IL-10 plays a key role in regulating the expression of the PD-1 ligands (36). Therefore, the interaction between both pathways cannot be discarded. Our results suggest a compensatory relationship between both pathways that might be reflected by the impaired parasite clearance observed during infection of PD-L1KO mice and are supported by reports where PD-L1 blockade promotes the induction of IL-10 (49). Additionally, a recent study on Toxoplasma infection revealed that the absence of PD-1 signaling promotes an increase in IL-10 production by $CD4^+$ and $CD8^+$ T cells, which increases the susceptibility to other opportunistic infections (14). Based on our results, it would be interesting to evaluate if the blockade of the IL-10/IL-10R pathway influences the level of PD-1 or PD-L1 expression. Due to the upregulation of PD-1 during the course of *T. cruzi* infection and the upregulation of Tim-3 upon PD-L1 deficiency, a combined blockade of PD-1 and Tim-3 using monoclonal antibodies was evaluated in infected *T. cruzi* WT mice. Our results showed that the combined blockade of

PD-1 and Tim-3 did not improve the course of infection. Neither the activation nor the functional capacity of T cells in comparison to infected isotype-treated control mice was found to be increased. Nevertheless, the combined blockade led to an increase of IL-10 and IFN-γ systemically. Thus, our hypothesis, that in *T. cruzi* infection, compensatory signaling through the Tim-3 pathway might be sustaining the potential dysfunction of T cells upon interruption of the PD-1 signaling could not be confirmed after the combined blockade experiments. The qRT-PCR results showed a trend, but not a statistically significant increase in parasite load in the analyzed tissues. This result is in agreement with a work in *T. cruzi* Sylvio X10/4 chronic infection where it was shown that blocking PD-1/PD-L1 interaction with monoclonal antibodies did not reduce the parasite load in the heart and the parasite persists due to a failure of the blockade to achieve an increased IFN-γ and TNF-α production by infiltrating T cells (8). In summary, our study shows that disruption of the PD-1/PD-L1 inhibitory pathway in the acute phase of the *T. cruzi* infection in mice does not provide increased protection against this parasite. In contrast, the interruption of the PD-1/PD-L1 pathway showed an increase in parasitemia as well as in the production of regulatory cytokines such as IL-10. This cannot be explained by enhanced induction of other co-inhibitory pathways such as Tim-3 in the absence of PD-1, as the dual blockade of these two pathways did not successfully enhance parasite elimination or T cell activation. It is tempting to speculate that other inhibitory pathways might favor persistence. Therefore, our results demonstrate that further research is urgently needed to better understand T-cell regulation in CD before immunotherapeutic approaches can be developed for a clinical setting, since the clinical use of immunotherapeutic approaches targeting the PD-1/PD-L1 axis in CD may be risky and associated with adverse events.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by The office of consumer protection in Hamburg, Germany (Application Nr. 52/17).

AUTHOR CONTRIBUTIONS

TJ developed the concept of the study, guided the writing process, and edited the final manuscript. YA and RIG performed the experiments, analyzed the data, and wrote the manuscript; All authors drafted the conclusion and provided critical feedback to shape the manuscript.

FUNDING

YA was supported by DAAD (Deutscher Akademischer Austausch Dienst) German Academic Exchange Service with Research Grant for Doctoral Students and Young Academics (500154434) Ref.# 91537601. RIG was a recipient of a Merit-based Ph.D. scholarship from the Konrad Adenauer Foundation (KAS), Germany.

ACKNOWLEDGMENTS

We thank the staff from the BNITM Animal Facility, Christiane Steeg and Ulricke Richardt for their excellent technical support. YA. expresses her sincere gratitude to the DAAD (Deutscher Akademischer Austausch Dienst) for their generous support during her stay in Germany. RG thanks the Konrad Adenauer Foundation (KAS, Germany) for the scholarship and support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.866120/full#supplementary-material

Supplementary Figure 1 | (A) Experimental set-up for the infection of BMDCs *in vitro.* **(B)** Flow cytometry gating strategy for BMDCs.

Supplementary Figure 2 | (A) Flow cytometry Gating strategy and exemplary dot plots for PD-L1 expression *in vivo* on $CD11c^+$ $CD11b^-$ cells (DCs) and $CD11b^+Ly6G^-Ly6C^+$ cells (MΦ) at day 15 post-infection. **(B)** Expression of PD-L2 on $CD11c^+CD11b^-$ (DCs) and, Ly6G-Ly6C+CD11b+ (MΦ). Data are a compilation of two independent experiments (n.i. = 4; infected=6). Data were analyzed for statistical significance using the Kruskal-Wallis test following Dunn´s multiple comparisons test. Asterisks denote P values as described in Methods; ns (not significant) were not plotted to avoid busy figures.

Supplementary Figure 3 | (A) Gating Strategy and exemplary dot blots for co-inhibitory receptors expression on $CD4^+$ and $CD8^+$ T cells from n.i. and infected WT and PD-L1 KO mice. **(B)** PD-1 on $CD4^+$ T cells **(C)** PD-1 on $CD8^+$ T cells. **(D)** Tim-3 on $CD4^+$ T cells **(E)** Tim-3 on $CD8^+$ T cells. **(F)** CD244 on $CD4^+$ T cells **(C)**. CD244 on $CD8^+$ T cells

Supplementary Figure 4 | (A) Parasitemia and **(B)** Bodyweight curves incl. the absolute number of WT and PDL1 KO mice used per timepoint. Data are from three independent experiments. Values are given as means ± standard error of the means (SEM). **(C)** Tim-3 induction is *T. cruzi* strain independent since it is significantly higher also after infection with the *T. cruzi* Brazil strain, which leads to chronic infections in mice.

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

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frontiers | Frontiers in Immunology

MINI REVIEW
published: 24 June 2022
doi: 10.3389/fimmu.2022.908034

Alternative B Cell Differentiation During Infection and Inflammation

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Long-term protective immunity to infectious disease depends on cell-mediated and humoral immune responses. Induction of a strong humoral response relies on efficient B cell activation and differentiation to long-lived plasma cells and memory B cells. For many viral or bacterial infections, a single encounter is sufficient to induce such responses. In malaria, the induction of long-term immunity can take years of pathogen exposure to develop, if it occurs at all. This repeated pathogen exposure and suboptimal immune response coincide with the expansion of a subset of B cells, often termed atypical memory B cells. This subset is present at low levels in healthy individuals as well but it is observed to expand in an inflammatory context during acute and chronic infection, autoimmune diseases or certain immunodeficiencies. Therefore, it has been proposed that this subset is exhausted, dysfunctional, or potentially autoreactive, but its actual role has remained elusive. Recent reports have provided new information regarding both heterogeneity and expansion of these cells, in addition to indications on their potential role during normal immune responses to infection or vaccination. These new insights encourage us to rethink how and why they are generated and better understand their role in our complex immune system. In this review, we will focus on recent advances in our understanding of these enigmatic cells and highlight the remaining gaps that need to be filled.

Keywords: atypical memory B cells, tissue-like B cells, double-negative B cells, T-bet, CD11c, FcRL5, CD21

OPEN ACCESS

Edited by:
Christine Hopp,
National Institutes of Health (NIH), United States

Reviewed by:
Juan L. Rivera-Correa,
NewYork-Presbyterian, United States

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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *30 March 2022*
Accepted: *30 May 2022*
Published: *24 June 2022*

Citation:
Courey-Ghaouzi A-D, Kleberg L and Sundling C (2022) Alternative B Cell Differentiation During Infection and Inflammation. Front. Immunol. 13:908034. doi: 10.3389/fimmu.2022.908034

INTRODUCTION

Vaccines constitute one of the most successful medical inventions known to date and have allowed the eradication or control of several previously common and deadly diseases. However, despite considerable efforts, vaccine development has proven difficult for some infections. For many of the vaccines, the best correlate of protection is humoral immune responses, derived from long-lived B cell memory, in the form of antibody-secreting plasma cells (ASCs) and memory B cells (MBCs) (1).

Long-lived B cell responses are generated following antigen encounter by naïve B cells and subsequent interactions with activated cognate CD4 helper T (Th) cells at the T-B border in secondary lymphoid organs (2). This initial extrafollicular interaction promotes B cell receptor (BCR) class-switching (3) and differentiation to either of several fates, such as early memory B cells, short-lived antibody-secreting cells (ASCs), or germinal center (GC) B cells in an antigen and affinity-dependent manner (4–6). B cells fated for GC selection enter the B cell follicles where they start to rapidly proliferate and form a dark zone and light zone GC structure (7). Within the GC, the B cells will undergo isotype-dependent selection (8) and affinity maturation to eventually

differentiate to long-lived MBCs and ASCs (9–12). The MBCs will then circulate between secondary lymphoid organs *via* the blood, or reside at sites of infection or inflammation (13), while the ASCs establish themselves in specialized niches that can sustain their extensive antibody production (14).

In addition to antibody production, B cells also have important roles as regulators of the immune response. Both by secreting inflammatory mediators, such as TNF-α and IL-6 (15), driving inflammation but also suppressing excessive inflammation through secretion of IL-10 and metabolizing extracellular ATP to ADP (16–18). Additionally, B cells are professional antigen-presenting cells (APCs) as they express high levels of MHC class II and can rapidly upregulate co-stimulatory molecules, such as CD80, CD86, and ICOSL upon stimulation. This interaction is important for subsequent B cell responses but also to drive T follicular helper (Tfh) cell differentiation at the B cell-T cell interfollicular region (19) and is proposed to promote effector T cell responses at sites of inflammation (20, 21).

In addition to the classical cell fates, B cells have also been shown to differentiate to an alternative B cell subset, often denoted as atypical, pro-inflammatory, exhausted, $CD27^{-}IgD^{lo}$ double negative, or tissue-like B cells, depending on the context in which they were identified (22). In this minireview, we further describe these cells in the context of different diseases or vaccination and highlight what is known about their origin, differentiation, migration and what potential function they might have within the immune response. As the nomenclature of these cells varies greatly between different studies, we have strived to use the most common overlapping markers used in the different papers.

ALTERNATIVE B CELL DIFFERENTIATION IN DISEASE AND VACCINATION

Resting naïve and memory B cells express complement receptor 2 (CD21), a co-receptor for the BCR (23), that is important in reducing the activation threshold upon BCR ligation (24). In 2002 Warnatz et al. described a B cell subset lacking CD21 expression in immunodeficient patients (25). Ehrhardt et al. reported in 2005 a similar B cell subset, lacking CD21 and the memory marker CD27 in different tissues and B cell lines (26). Although the markers used to distinguish this alternative B cell subset have not been firmly established, expression of the transcription factor T-bet, the integrin CD11c, different Fc receptor-like (FcRL) proteins, and chemokine receptors (such as CXCR3) have been used (27). This subset of B cells is present in healthy individuals at low levels (28) and increases with age (29), but also expands greatly during inflammatory conditions, including infections, autoimmune disorders, and after vaccination (30, 31).

In 2009 Weiss et al. reported the expansion of a subpopulation of B cells amongst people living in malaria-endemic areas (32). The B cells were referred to as atypical memory B cells and were identified through the low expression of CD21 and CD27 (32). Several additional studies have since shown that $CD21^{lo}CD27^{lo}$ B cells greatly expand during infection with malaria parasites (33–38). Tissue-like memory B cells, which have a similar $CD21^{lo}CD27^{lo}$ phenotype as the atypical memory B cells, but also express FcRL4 are described to expand in individuals infected with human immunodeficiency virus (HIV) (39–41). The cells showed reduced BCR signaling and antibody production upon stimulation, leading to the thought that the B cells were anergic or exhausted (39). Other infections where B cells with a $CD21^{lo}CD27^{lo}IgD^{-}$ phenotype are observed to expand are hepatitis C virus (HCV) (42, 43), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (44–46), hantavirus infection (47) tuberculosis (48), and possibly others.

In the autoimmunity field, B cells lacking CD27 and IgD, often termed double negative (DN) B cells, are seen to increase in various diseases with inflammatory components. In systemic lupus erythematosus (SLE), the DN B cells express CD11c, FcRL5 and T-bet (49, 50), markers largely overlapping with those expressed during infection. Similar $CD21^{lo}CD27^{lo}CD11c^{+}$ B cells have also been reported to expand in a subset of in patients with common variable immunodeficiency (CVID), rheumatoid arthritis (51) and multiple sclerosis (52). This phenotypic overlap is further supported by largely overlapping transcriptional programs based on bulk microarray and RNA sequencing of $CD11c^{hi}$ cells (53) and single-cell RNA sequencing (54).

MECHANISMS OF ALTERNATIVE B CELL DIFFERENTIATION

Although initial observations of expanded $CD21^{lo}$ B cell numbers were primarily in the context of chronic immune activation, more recent studies in both mice and humans have shown that $CD21^{lo}$ T-bet^{+} or $CD11c^{+}$ B cells expand rapidly after infection or immunization (36, 41, 45, 55), after which they slowly decline over several months (36, 38, 41, 56) (**Figure 1**). Similarly, these cells start to decline after treatment of individuals with HCV (43) and tuberculosis (48), indicating that the cells need a sustained proinflammatory environment and or antigen-stimulation to survive.

In malaria, the expansion of $CD21^{lo}CD27^{lo}$ B cells is associated with the intensity of parasite transmission (33), consistent with antigen exposure in a proinflammatory environment being important in driving expansion or survival of these cells (32, 57, 58). Studies have linked the expansion of T-bet^{+} B cells to the pro-inflammatory cytokine interferon-γ (IFN-γ), both in the context of malaria (59) and SLE (50, 60). IFN-γ is a T helper type 1 (Th1) cytokine, which upon binding to the IFN-γ receptor on B cells activates the JAK-STAT signaling pathway, resulting in up-regulation of the transcription factor T-bet (61), which is important for IgG2a/c class-switching in mice (62) and likely IgG3 in humans. In contrast to T-bet, upregulation of CD11c seems to require BCR-ligation but not IFN-γ (63), potentially explaining why these proteins are not always co-expressed. The differentiation to $CD21^{lo}$ T-bet^{+} or $CD11c^{+}$ B cells has primarily been described to start from classical MBCs (34, 64). However,

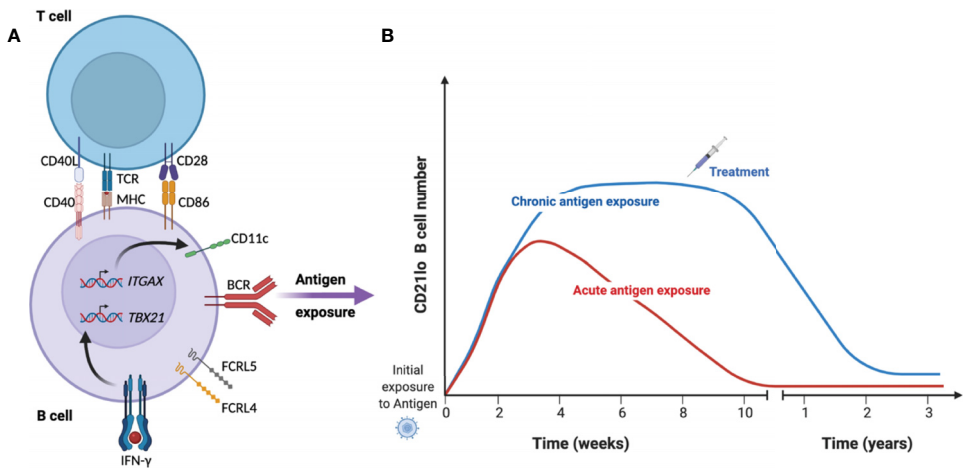

FIGURE 1 | Kinetics of CD21^{lo} CD11c^{+} T-bet^{+} B cells during acute and chronic disease. **(A)** Under specific conditions such as B cell receptor (BCR) activation in the presence of IFN-γ and T cell help, B cell will acquire a phenotype characterized by reduced expression of CD21 and increased expression of CD11c (*ITGAX*), T-bet (*TBX21*) and FCRL4/5. **(B)** They then rapidly expand over several weeks until they start to contract over several months in the absence of antigen (red line). In the context of chronic infection or repeated infection (blue line), the cells can remain a substantial proportion of total B cells until treatment removes the source of antigen and reduce the inflammatory response.

many of the CD21^{lo} T-bet^{+} or CD11c^{+} B cells display an unswitched BCR (36), potentially indicating a naïve B cell origin. In support of this, BCR sequencing shows partly overlapping repertoire and gene characteristics between naïve B cells and unswitched CD21^{lo}CD27^{lo} B cells (65, 66). Further, Obeng-Adjei et al. reported that Th1 and Tfh-1 cells could induce a T-bet^{hi} phenotype in naïve B cells after two days of co-culture together with the superantigen staphylococcal enterotoxin B (59).

Ambegaonkar et al. further investigated which stimuli lead to upregulation of T-bet in naïve, GC, or memory B cell subsets *in vitro* (67). They found that BCR-ligation together with IFN-γ and the TLR9-ligand CpG could effectively make naïve and memory B cells, but not GC B cells, upregulate T-bet in addition to other surface proteins associated with CD21^{lo} B cells. Interestingly, naïve B cells were more capable of upregulating T-bet compared with MBCs under the conditions tested (67). Keller et al. investigated the contribution of different signaling pathways important for the generation of CD21^{lo} cells *in vivo* by studying individuals with CVID (60). They observed that BCR ligation together with CD4^{+} T cell-derived CD40L, IFN-γ, and IL-21 are important for the expansion of CD21^{lo}T-bet^{hi} B cells, thus proposing that the expansion of these cells *in vivo* is T cell-dependent (60). This is also consistent with recent studies in mice, where CD11c^{+}T-bet^{+} B cells generated after viral or intracellular bacterial infection require interactions with Tfh cells (68, 69).

ROUTE OF DIFFERENTIATION

The more efficient upregulation of T-bet in naïve and memory B cells could indicate that CD11c^{+}T-bet^{+} B cells are primarily generated *via* the extrafollicular route, rather than *via* the GC. Both extrafollicular and GC B cells can undergo class-switch recombination and somatic hypermutation (70, 71), although the extrafollicular response is more rapid and associated with an expansion of ASCs, further reviewed by Elsner and Schlomchik (72). Jenks et al. point to several features of CD11c^{+} DN2 B cells, found in SLE patients, associated with extrafollicular differentiation, such as the cells lacking expression of CXCR5, a chemokine receptor involved in migration to secondary lymphoid organs, and CD62L, important for the trafficking to lymph nodes (73). Such receptor expression has also been described for CD11c^{+} B cells during malaria and hepatitis infection (32, 74). Additionally, sequencing of BCRs showed a similar mutation level of IgG in DN2 cells and ASCs, but lower than for switched memory B cells, suggesting that the DN2 cells had not gone through the GC while sharing common developmental pathways with ASCs (73). However, in several other studies associated with infection or vaccination, the mutation level was similar between conventional memory B cells and CD11c^{+}Tbet^{+} B cells (64, 75, 76). Drawing strong conclusions based on BCR sequence analysis can potentially be misleading since it is possible that the CD11c^{+}T-bet^{+} cells originate from conventional MBCs that could have a GC origin (34, 64). Therefore, investigating unswitched CD11c^{+}T-bet^{+} B cells with a likely naïve origin could provide more direct support for the extrafollicular route, as these cells are more unlikely to have entered a GC reaction.

Contrasting with an extrafollicular route of CD11c^{+}T-bet^{+} B cell differentiation, several studies have identified T-bet^{+} B cells in ongoing GCs in mice after challenge with malaria parasites (77) or influenza virus (75). Similar to peripheral T-bet^{+} B cells, GC B cells also have reduced levels of CD21 but few studies present data on CD11c expression among GC B cells. CD21^{lo}CD27^{+} B cells in human peripheral blood that also have

reduced CXCR5 and CD62L and high expression of Fas, similar to the DN2 B cells, were suggested to have a GC origin by Lau et al. (55). This conclusion was, however, largely based on BCR sequence analysis and mutational evolution after vaccination.

Although it is attractive to say that CD11c$^+$T-bet$^+$ B cells only have one origin and differentiation route, the extrafollicular and GC pathways are not mutually exclusive. Furthermore, it is possible that the conditions of the inflammatory response largely determine the route. This has been further discussed by Elsner and Schlomchik (72), where they propose that high levels of IFN-γ suppresses Tfh development and subsequent GC responses, promoting differentiation *via* the extrafollicular route, while lower IFN-γ levels can allow for Tfh-mediated T-bet$^+$ GC B cell differentiation. However, It remains difficult to formally prove which route the peripheral CD11c$^+$T-bet$^+$ B cells took upon differentiation, especially in humans, where fate mapping approaches are not possible.

ASSOCIATION WITH ANTIBODY-SECRETING CELLS

Using RNA sequencing, Wang et al. noted that CD11c^{hi} B cells in SLE had upregulated genes associated with ASC differentiation, such as *PRDM1* (Blimp-1), *AICDA* (AID), *XBP1, BMP6*, *EMP3*, and *S100A4* (49). Furthermore, after culturing CD11c^{hi} B cells together with anti-CD3-activated T cells for 11 days, 70% of the cells expressed a CD27$^+$CD38^{hi} ASC phenotype (49). Consistent with this, Golinski et al. also found that a larger proportion of CD11c$^+$ B cells differentiated into ASCs compared to CD11c$^-$ B cells, in addition to secreting more IgM and IgG after 7 days of culture in the presence of BCR ligation, TLR9-ligand, and IL-21 (63). These observations contrast with previous reports on restimulation of CD21^{lo} B cells in malaria, HIV, and Hepatitis B, where the cells displayed reduced differentiation to ASCs compared with classical memory B cells (34, 35, 39). This could potentially be due to intrinsic differences in the cells associated with autoimmune versus infectious diseases. But it could also be associated with the experimental conditions and the cell types included in the analysis, where a CD11c sort would likely include more CD21$^+$ resting memory B cells than the sort for CD21^{lo}CD27^{lo} B cells. However, consistent with the CD11c^{hi} transcriptomic data (49), Hopp et al. also found the ASC-associated genes *PRDM1* and *CD38* upregulated in CD21^{lo}CD27^{lo} B cells during acute malaria (78). Furthermore, restimulation of sorted CD21^{lo}CD27^{lo} B cells with superantigen activated Tfh cells led to ASC differentiation with upregulation of CD38 and production of IgG and IgM antibodies (78). This indicates that these cells can differentiate to ASC although the process could be context-dependent, such as the availability of Tfh cells.

POTENTIAL FUNCTIONS OF CD21^{lo} B CELLS

Although CD21^{lo} B cells can represent up to 50% of the circulating B cells in people living in malaria-endemic areas (32–34, 79) and are generated rapidly after vaccination or infection and have been proposed to be a normal part of the immune response (80, 81), the potential function of these cells remains largely unclear. Based on increased cell surface expression of several inhibitory receptors, such as CD22, CD85j, and FcγRIIB, and reduced responsiveness to restimulation of sorted human CD21^{lo} FcRL5$^+$ or FcRL4$^+$ B cells, these cells have been hypothesized to be exhausted or dysfunctional (34, 35, 39). Muellenbeck et al. showed that the cells were enriched for self- or polyreactive BCR specificities (76), potentially indicating that they could have been made anergic to protect the host from autoimmunity. However, Muellenbeck et al. also found BCR specificities of the cells overlapping with antibodies in plasma and mRNA transcripts corresponding to secretory antibodies, suggesting that the cells could contribute to the circulating antibody pool (76). This potential role has been further substantiated by findings that T-bet$^+$ B cells can produce antibodies binding to phosphatidylserine on red blood cells, possibly contributing to anemia during malaria (82, 83), and similarly able to produce self-reactive antibodies in human and mouse models of SLE (73, 80, 81).

A recent study by Ambegaonkar et al. indicates a mechanism of how CD21^{lo}CD27^{lo} B cells can come across as dysfunctional in restimulation assays, and as important contributors to the secreted antibody pool in other studies (84). They show that the high expression of inhibitory receptors, and especially FcγRIIB, restricts CD21^{lo}CD27^{lo} B cells in their response to soluble antigen. However, in conditions where the BCR ligand or antigen is presented to the cells while fixed in a lipid bilayer, FcγRIIB is excluded from the immunological synapse, allowing the engagement of CD19 with the BCR (84). Such conditions, summarized in **Figure 2**, induce a strong BCR signal leading to the expression of IRF4, which is associated with ASC differentiation (85).

Experiments in mice have indicated that CD11c$^+$T-bet$^+$ B cells are associated with protection from chronic viral infection (86). However, this effect was not only associated with antibody production but also with other cell-mediated mechanisms. In addition to their potential role as ASC precursors, CD21^{lo} B cells upregulate proteins with important functions in the T-B synapse, such as MHC class II and the co-stimulatory molecules CD80, CD86, OX40, and ICOS-L (78, 80). They are also more efficient than naïve and conventional memory B cells in taking up antigens (84). Together this could indicate that the cells have become more potent as APCs. Indeed, CD11c$^+$ B cells in mice were able to improve CD4$^+$ T cell activation and proliferation compared with follicular B cells (87, 88) and depletion of CD11c$^+$ B cells in mice led to reduced Tfh cell levels (88). In contrast, sorted human CD21^{lo} B cell subsets provided similar CD4$^+$ T cell activation as CD21$^+$ B cell subsets in an *in vitro* mixed-lymphocyte reaction assay (89). However, since CD11c$^+$ and T-bet$^+$ B cells also upregulate homing receptors, such as CXCR3, they can migrate to sites of inflammation and provide localized APC functions or potentially complete differentiation to ASCs. Such an effect was recently described by MacLean et al. where they showed that CXCR3$^+$ lung-resident memory B cells were

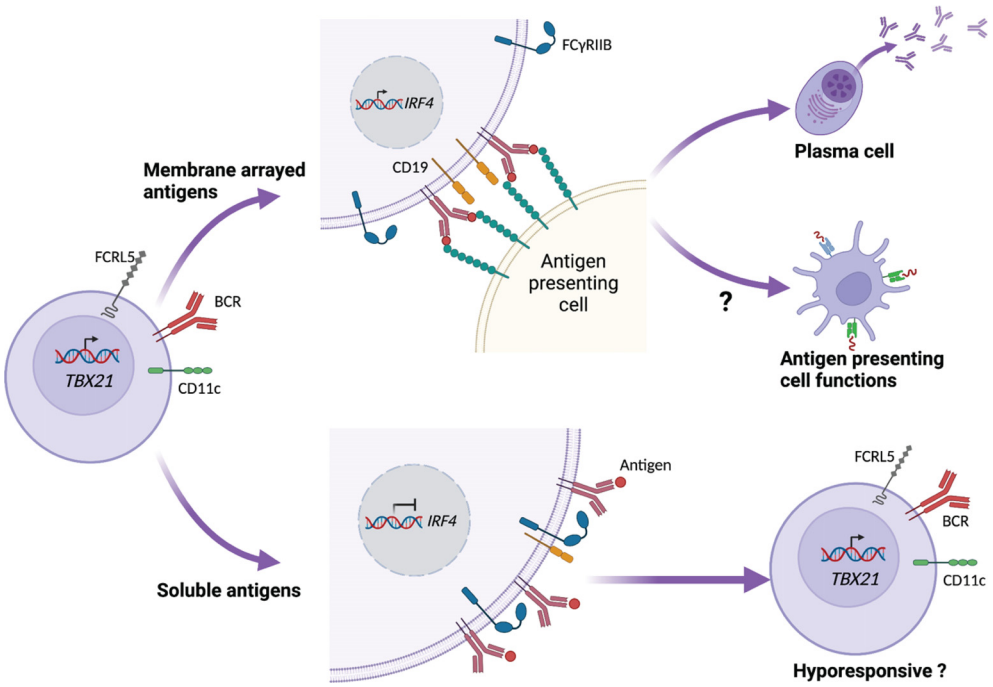

FIGURE 2 | CD21^{lo} B cell responsiveness is dependent on how antigen is presented. CD21^{lo} B cells expressing CD11c, T-bet (*TBX21*), and FCRL5 also express increased levels of inhibitory receptors, such as FcγRIIB. In the context of B cell receptor (BCR) ligation, FcγRIIB reduces the engagement of CD19 with the BCR, thus preventing down stream signaling, leading to hyporesponsiveness. In contrast, CD21^{lo} B cells binding to membrane arrayed antigens establish an immunological synaps that excludes FcγRIIB. This allows CD19 to engage the BCR and promote downstream signaling, leading to transcription of *IRF4* that in turn can drive differentiation to antibody secreting cells. The membrane-arrayed antigens also lead to improved antigen-uptake and could potentially enhance the B cell antigen-presenting cell functions.

recruited to infected foci in the lung in an IFN-γ dependent manner upon reinfection of mice with influenza virus. The cells then differentiated to ASCs at the foci to provide localized antibody production (90).

Overall, these studies highlight that CD21^{lo} CD11c^{+} or T-bet^{+} B cells should no longer be considered as an exhausted or dysfunctional B cell subset, but rather as fully capable of responding to specific signals. Although the extent of the role they play in a systemic immune response to infection such as malaria or during autoimmune disease remains largely unclear, recent studies indicate important functions that need to be further explored.

LOCALIZATION AND HOMING OF CD21^{lo} B CELLS

One of the many unanswered questions regarding CD21^{lo}T-bet^{+} B cells is the localization and homing of such cells. A recent study by Johnson et al., investigating T-bet^{+} B cells in patients undergoing surgery and in mice, showed a similar pattern of distribution in different tissues (75). They also showed that influenza-specific T-bet^{+} B cells were differentially distributed in the spleen, peripheral blood, bone marrow, and lung, indicating that the cells had a preferred tissue homing associated with the infection.

Interestingly, only B cells expressing low levels of T-bet were present in the lymphoid circulation, while T-bet^{hi} B cells were absent from lymph nodes (75). This is consistent with previous studies (26, 91), where B cells lacking CD27 and expressing FCRL4 or FCRL3 were present in lymph nodes, tonsil, and payer patches, but few, if any cells expressing high levels of T-bet (91). Similarly, after influenza vaccination in humans, B cells expressing *TBX21, FCRL5*, and *ITGAX* were present in peripheral blood, but not in the draining lymph nodes (92). This partial or absent expression of markers associated with CD11c^{+}T-bet^{+} B cells could indicate that differentiation concurrently changes the receptor expression to promote migration from secondary lymphoid organs to tissues, consistent with low levels of CD62L, CXCR5, and CCR7 on these cells in the blood (32, 73). It is however interesting to note that CD11c^{+} T-bet^{+} B cells are present in the spleen but not in lymph nodes (75). In mice, it was recently described by Song et al, that CD11c^{+}T-bet^{+} B cells generated after virus infection were retained in the splenic marginal zone through interactions by LFA-1 and VLA-4, indicating a potential mechanism of splenic retention (68), while the mechanisms of homing to and retention in other tissues remain to be explored. Further studies are also needed to understand how differentiation is associated with migration.

CONCLUSION

Upon immune activation, B cells differentiate to provide the host with several important effector functions. Of these, antibody production is without a doubt the most well described, but B cells also provide important roles as APCs in addition to secret different cytokines to both promote and suppress inflammatory responses (15, 93).

Over the last decade, a phenotypically distinct, although heterogenous B cell subset, identified through reduced expression of CD21 and CD27 and upregulation of one or several of T-bet, CD11c, and FcRL4/5, has gained increasing attention. In this review, we have presented recent data generated in different research fields from human samples and mice, and although the inconsistent use of names and markers to identify these cells often makes direct comparisons difficult, several studies point toward these cells having largely overlapping phenotypic and transcriptional signatures and homing patterns. However, many studies also point to substantial heterogeneity in the markers expressed by these cells between diseases and over time and also between mice and humans. This illustrates that further studies that directly compare the cells between diseases, time-points, tissues, organisms, or stimulations using the same systematic approach are needed. Such comparative studies would also be very useful for the research community to decide on a more systematic nomenclature for these cells.

AUTHOR CONTRIBUTIONS

All listed authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication. ADCG and LK contributed equally to the work and the author order was determined by dice.

FUNDING

This work was supported by grants from the Swedish Research Council (2019-01940), Åke Wiberg foundation (M18-0076), Magnus Bergvall Foundation (2017-02043 and 2018-02656), Tore Nilsson Foundation (2018-00608), and Karolinska Institutet Faculty funds (2020-00878) to CS. None of the sources of funding has an interest in the subject matter or materials discussed in the submitted manuscript. ADCG is supported by Karolinska Institutet PhD program faculty funds.

ACKNOWLEDGMENTS

We thank all the lab members that have read and commented on the manuscript. The figures were created with BioRender.com

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

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 29 June 2022
DOI 10.3389/fimmu.2022.864632

OPEN ACCESS

EDITED BY:
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SPECIALTY SECTION:
This article was submitted to
Parasite Immunology,
a section of the journal
Frontiers in Immunology

RECEIVED 28 January 2022
ACCEPTED 14 June 2022
PUBLISHED 29 June 2022

CITATION:
Leal-Silva T, Lopes CA,
Vieira-Santos F, Oliveira FMS,
Kraemer L, Padrão LLS, Amorim CCO,
Souza JLN, Russo RC, Fujiwara RT,
Magalhães LMD and Bueno LL (2022)
IL-17RA receptor signaling contributes
to lung inflammation and parasite
burden during *toxocara canis* infection
in mice.
Front. Immunol. 13:864632.
10.3389/fimmu.2022.864632

IL-17RA receptor signaling contributes to lung inflammation and parasite burden during *Toxocara canis* infection in mice

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IL-17 is a cytokine produced by innate and acquired immunity cells that have an action against fungi and bacteria. However, its action in helminth infections is unclear, including in *Toxocara canis* infection. Toxocariasis is a neglected zoonosis representing a significant public health problem with an estimated seroprevalence of 19% worldwide. In the present study, we describe the immunopathological action of IL-17RA in acute *T. canis* infection. C57BL/6j (WT) and IL-17RA receptor knockout (IL-17RA-/-) mice were infected with 1000 *T. canis* eggs. Mice were evaluated 3 days post-infection for parasite load and white blood cell count. Lung tissue was harvested for histopathology and cytokine expression. In addition, we performed multiparametric flow cytometry in the BAL and peripheral blood, evaluating phenotypic and functional changes in myeloid and lymphoid populations. We showed that IL-17RA is essential to control larvae load in the lung; however, IL-17RA contributed to pulmonary inflammation, inducing inflammatory nodular aggregates formation and presented higher pulmonary IL-6 levels. The absence of IL-17RA was associated with a higher frequency of neutrophils as a source of IL-4 in BAL, while in the presence of IL-17RA, mice display a higher frequency of alveolar macrophages expressing the same cytokine. Taken together, this study indicates that neutrophils may be an important source of IL-4 in the lungs during *T. canis* infection. Furthermore, IL-17/IL-17RA axis is important to control parasite load, however, its presence triggers lung inflammation that can lead to tissue damage.

KEYWORDS
IL-17RA, toxocariasis, cytokines, helminth, inflammation, neutrophil

Introduction

IL-17 is an inflammatory cytokine produced by several innate and adaptive immune cells such as Th17 lymphocytes, CD8 T cells, natural killer cells, γδ T cells, macrophages, neutrophils, eosinophils, and innate lymphoid cells (ILCs) (1–3). IL-17 family cytokines mediate effects at the molecular level by binding to their receptors, known as IL-17R family receptors (IL-17RA - IL-17RE), which have unique structural characteristics and trigger signaling events (4). IL-17A and IL-17F are homonymous cytokines of the Th17 lineage, which can form IL-17A–IL-17F heterodimers and are the best characterized cytokines of the IL-17 lineage. The cytokines IL-17A, IL-17F and IL-17A–IL-17F signal through the same receptor subunits, IL-17RA and IL-17RC, which together form a heteromeric complex (4, 5). The inflammatory capacity of IL-17 is related to the recruitment of immune cells such as neutrophils and monocytes and synergistic action with other cytokines such as TNF, IL-1β, IFN-γ, GM-CSF, IL-22 (5).

The IL-17 presents an important response against extracellular bacteria and fungi (5). However, the role of IL-17 in helminth infections is still controversial and appears to be pathogen-specific. Studies in animal models infected with *Schistosoma* spp. indicated that IL-17 contributed to the pathogenesis of liver fibrosis and increased granulomatous inflammation in the lung and liver (6–8). In *Ascaris suum* infection, the systemic polarized Th2/Th17 immune response appears to be crucial to control larval migration after multiple exposures to *Ascaris* (9). Using a model of lung infection by *Nippostrongylus brasiliensis*, it was reported that IL-17 and neutrophilic inflammation limited parasite survival but caused increased lung injury (10). Furthermore, it was demonstrated that IL-4R signaling controlled IL-17 elevations, increased IL-10 and stimulated the development of M2 cells, contributing to the resolution of tissue damage, showing that the Th2 response can contribute to the acute healing of wounds during helminth infection (11). Another study reported that IL-17A is an important regulator of type 2 pulmonary immunity in *N. brasiliensis* infection. IL-17A supports the development of type 2 response through IFN-γ suppression, however, in the later phase, IL-17A limited excessive type 2 responses, proposing a feedback mechanism (12). In *Toxocara canis* infection, studies have shown that IL-17 is increased in serum and lung tissue, but its role during infection has not yet been elucidated (13, 14).

Toxocariasis is a neglected zoonosis whose principal etiological agent is *T. canis*. It is estimated that the worldwide seroprevalence of toxocariasis is around 19%, with the highest rates being associated with countries with higher temperature and humidity, lower-income levels, and lower human development index (11, 15). Humans become infected by accidentally ingesting eggs containing the infecting larvae, hatching the intestine, penetrating the intestinal mucosa, and migrating to various organs, including the lungs, where they trigger an inflammatory response with eosinophilia and increased production of cytokines and specific antibodies (16–18).

In recent years, the number of studies on the immune response triggered by *T. canis* has increased (9, 10, 13, 14), however, the immunological mechanisms involved in protection and injury during infection are still poorly understood. In this context, the present study aimed to describe the immunopathological role of IL-17RA in acute *T. canis* infection. Our results revealed that IL-17/IL-17RA axis is important for controlling the pulmonary larval load, and this fact may be related to the increase in IL-6 and the frequency of IL-4 producing neutrophils. However, its presence increases pulmonary inflammation and can trigger lung tissue damage.

Methods

Parasites

Adult *T. canis* worms were collected from feces of naturally infected puppies treated with anthelmintics (Drontal Puppy, São Paulo, Brazil) at a dosage of 1mL/kg. Puppies were kept at the Zoonoses Control Center in Belo Horizonte, Minas Gerais, Brazil. Adult worms were kept in water and taken to the Laboratory of Immunology and Genomics of Parasites at the Federal University of Minas Gerais to be processed. Eggs were isolated from the uterus of adult female worms by mechanical maceration, purified by filtration on 100 μm nylon filters, placed in culture flasks with 50 mL of 0.2 M sulfuric acid, and kept in a BOD incubator at 26°C. After 6 weeks of cultivation, at the peak of larval infectivity, fully embryonated eggs were used for experimental infections (13).

Mice

Female C57BL/6j or IL-17RA$^{-/-}$ mice at approximately 8 weeks of age were used for this study. Mice genetically deficient for the IL-17RA receptor (IL-17RA$^{-/-}$) were acquired at the Special Mouse Breeding Center of the Faculdade de Medicina de Ribeirão Preto (USP), and wild-type mice (WT) C57BL/6j were obtained from the animal facility of the Federal University of Minas Gerais. During the experimental period, mice were fed with filtered water and commercial chow (Nuvilab Cr-1, Nuvital Nutrients, Brazil) ad libitum. Mice were maintained at the Animal Facility of the Department of Parasitology of the Federal University of Minas Gerais under controlled temperature conditions (24 ± 1°C) and lighting (12-hour light-dark cycle).

Experimental design and *T. canis* infection

WT and IL-17RA$^{-/-}$ mice were randomized and sacrificed on day 0 (control group), 3, 14, and, 63 days post-infection (dpi), as shown in **Figure 1**. Fifty mice of each strain were used for performing all methodologies, distributed as follows: 6 mice for

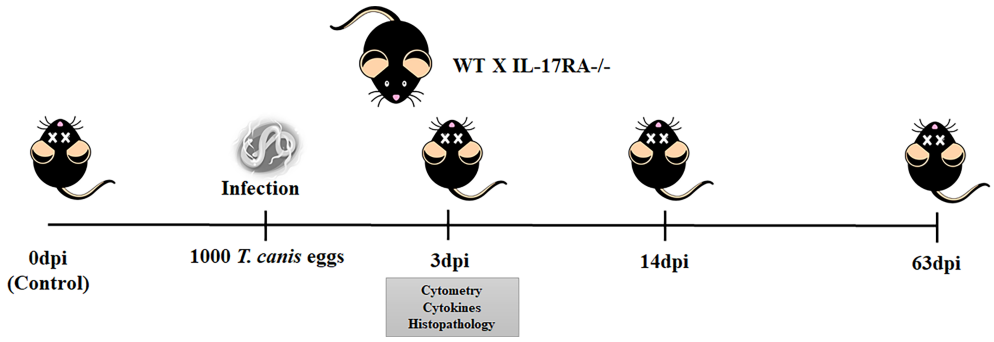

FIGURE 1
Experimental design of *T. canis* infection in mice. WT and IL-17RA-/- mice were infected with 1000 eggs of *T. canis* and euthanized with 0dpi, 3dpi, 14dpi, and 63dpi. With 3dpi, cytometry, cytokine, and histopathology analysis were performed.

each dpi to assess parasite burden and leukocyte profile (0dpi, 3dpi, 14dpi, and 63dpi), 6 mice for flow cytometry (0dpi and 3dpi), and 7 mice for histopathology and cytokine evaluation (0dpi and 3dpi). All mice were sacrificed with a lethal injection of xylazine/ketamine (8.5 mg/kg and 130 mg/kg).

For *T. canis* infection, prior to inoculation, embryonated eggs were incubated with 5% (v/v) sodium hypochlorite solution in an incubator (37°C and 5% CO_2) for 1 hour and 40 minutes to break up the outer layer of eggs and, therefore, to facilitate the hatching of larvae *in vivo*. After incubation, eggs were resuspended and washed with PBS 3 times. Mice from infected groups were inoculated by oral gavage with 0.2 ml of the solution containing 1000 embryonated eggs.

Parasitological analysis

Parasite burdens of infected mice were assessed by counting the total number of larvae recovered in the liver, lung, and brain with 3dpi, 14dpi, and 63dpi. Each tissue was collected, punctured with surgical scissors, and placed in a modified Baermann apparatus for 4 h in PBS at 37°C and 5% CO_2. The larvae recovered in the pellet of the apparatus were fixed in 4% formalin and quantified by light microscopy (14).

Leukocyte analysis

500μL of blood was collected by cardiac puncture and transferred to tubes containing the anticoagulant EDTA (Vacuplast, Brazil). Global leukocyte counts were performed using a Bio-2900 Vet automated hematology counter. Blood smears were stained with Panotic (Laborclin, Brazil) for differential leukocyte counts, and 100 leukocytes were differentiated under a light microscope.

Protein and hemoglobin in bronchoalveolar lavage

Mice were anesthetized, and a 1.7 mm catheter was inserted into the trachea. One milliliter of PBS was washed twice through the catheter to collect the bronchoalveolar lavage fluid. The lavage fluid was centrifuged at 3000 g for 10min, and the supernatants were used for hemoglobin and total protein quantification.

Hemoglobin was quantified to assess alveolar hemorrhage present in the BAL using the Hemoglobin K023-1 kit (Bioclin Quibasa, Brazil). Concentration was determined spectrophotometrically by measuring absorbance at 540 nm. Hemoglobin (Hb) concentration was expressed in g/dL of Hb per ml of BAL. Quantitation of total protein was determined by the BCA Protein Assay kit (Thermo Scientific, USA). Results were expressed as μg of total protein per ml of BAL.

Cytokine profiles

Pulmonary cytokines were evaluated at 3dpi in infected and uninfected mice. For that, the right lung of each animal was removed and homogenized (TissueLyser LT- Qiagen, German) in extraction solution (0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 IU aprotinin A) at a rate of 1 mL per 100 mg of lung tissue. The homogenates were centrifuged at 800 × g for 10 min at 4°C, and the supernatant was collected and stored at -80°C for cytokine quantification. Levels of TNF-α, IL-1β, IL-6, IL-12/IL-23p40, IL-17A, IL-4, IL-5, IL-33, IL-13, IL-10, and TGF-β were tested by an ELISA sandwich kit (R&D Systems, USA) according to the manufacturer's instructions. The absorbance of the samples was determined in a Versa Max ELISA microplate reader (Molecular Devices, USA) at a wavelength of 492 nm.

Histopathological analysis

The left lobe of the lungs was removed from mice infected with 3dpi and from uninfected 0dpi. The organs were fixed in a 4% formalin solution, gradually dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks that were cut 4–5 micrometers thick and fixed on a microscope slide. Lung tissue slides were stained with hematoxylin and eosin. All histopathological analyses were performed blindly.

For the airway inflammation score, 10 random images per animal were captured at 20X magnification as described in **Supplemental Table 1** and analyzed for perivascular inflammation, peribronchial inflammation, parenchymal damage, hemorrhage, and nodular aggregates formation.

Phenotypic analysis by flow cytometry

Multiparametric flow cytometric analyses were performed in BAL and peripheral blood of WT and IL-17RA$^{-/-}$ mice uninfected (0dpi) and infected with 3dpi to characterize the cell population and observe the cytokines secreted by each cell type.

In the BAL, all the following Bioscience® markers were used: CD4 (Fitc, clone H129.19, 1:100), IL-4 (PE, clone 11B11, 1:50), Siglec F (PeTxRed, clone E50-2440, 1:400), CD45 (PeCy7, clone 30-F11, 1:800), CD11c (APC, clone N418, 1:800), IL-17 (APCCy7, clone TC11-18H10, 1:50), I-A/I-E (MHCII) (BV480, clone M5/114.15.2, 1:50) IL-10 (BV421, clone JES5-16E3, 1:50), Ly6G (BV570, clone 1A8, 1:200).

In peripheral blood, two panels were used, in the first, the following Bioscience® markers were used: CD69 (Fitc, clone H1.2F3, 1:500) IL-4 (PE, clone 11B11, 1:50), Siglec F (PeTxRed, clone E50-2440, 1:400) CD4 (PeCy5, clone RM4-5, 1:800), CD27 (PeCy7, clone LG3A10, 1:200), CD8 (APC, clone 53-6.7, 1:800), IL-17 (APCCy7, clone TC11-18H10, 1:50), Viability (Fixable viability stain 700, 1:1000), I-A/I-E (MHCII) (BV480, clone M5/114.15.2, 1:50), TNF-α (BV421, clone MP6- XT22, 1:200), Ly6G (BV570, clone 1A8, 1:200).

In the second panel for monocyte identification, the following Bioscience® markers were used: I-A/I-E (MHCII) (Fitc, clone AF6-120.1, 1:50), Ly6C (PE, clone AL-21, 1:800), CD11b (PeCy5, clone M1/70, 1:200), TNF-α (BV421, clone MP6-XT22, 1:200), CX3CR1 (BV605, clone SA011F11, 1:200), Viability (Fixable viability stain 700, 1:1000).

BAL and peripheral blood cells were incubated with brefeldin (BD Biosciences®) at a concentration of 1μg/ml at 5% CO_2 for 4 hours at 37°C. After brefeldin incubation, cells were incubated with ACK lysis buffer for 10 minutes (1x for BAL and 3x for peripheral blood). After each lysis cycle, cells were washed with PBS and centrifuged at 300 G, 8 minutes, 4°C. Once the samples were free of erythrocytes, cells were counted in Neubauer chamber using Trypan blue and incubated with the viability dye (BD Bioscience®, Fixable viability stain 700, 1:1000) for 10 min at 4°C. After incubation, cells were washed by centrifugation at 300 G, 8 minutes, 4°C PBS. After Live/Dead staining, cells were incubated with a 20μl mix of surface antibodies containing 10% normal mice serum for 15 minutes at 4°C and washed with PBS by centrifugation at 300 G, 8 minutes, 4°C. Samples were then fixed with 2% PFA for 20min at room temperature. After fixation, cells were washed twice with PBS and permeabilized by incubation for 15min with a 0.5% saponin solution and proceeded to intracellular staining. 40μl of intracellular antibody mix were incubated for 30 min at room temperature. After intracellular staining, cells were washed twice with 0.5% saponin solution and resuspended in PBS. Samples were acquired in LSR Fortessa (BD Biosciences, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR). Data analysis was followed by dimensionality reduction and visualization by t-Distributed Stochastic Neighbor Embedding (tSNE) using Cytofkit (23) and rPhenograph to group cells into clusters according to their similarity of activation/expression molecules and cytokines and rPhenograph to group cells into clusters according to their similarity of activation/expression molecules and cytokines. Each experimental group in tSNE analysis represent 6 mice with input of the same number of cells. The gating strategy is illustrated in **Supplementary Figure 1** and the summary of all markers used in flow cytometry are in the **Supplemental Table 2**.

Statistical analysis

Statistical analysis was performed using Prism 8.0 software (GraphPad Inc, USA). Initially, the Grubbs test was used to detect possible outlier values. Followed by the Shapiro-Wilk test was performed to assess the normality of the variables. The comparison between two groups was performed using the Student's T test or the Mann-Whitney test according to the normality test. The evaluation between three or more groups was performed using the Analysis of Variance (ANOVA) or Kruskal-Wallis test, followed by Tukey's or Dunn's post-test, respectively, according to the data distribution. In all analyses, the results with a value of $p<0.05$ were considered statistically significant.

Results

The absence of the IL-17RA receptor increases the pulmonary parasite load and induces leukocytosis

To assess the response of IL-17RA in *T. canis* infection, the larvae recovery in the liver, lungs, and brain tissues was performed (**Figures 2A–C**). A significant increase in larvae in lung tissue after 3dpi was observed in IL-17RA$^{-/-}$ mice compared to WT mice.

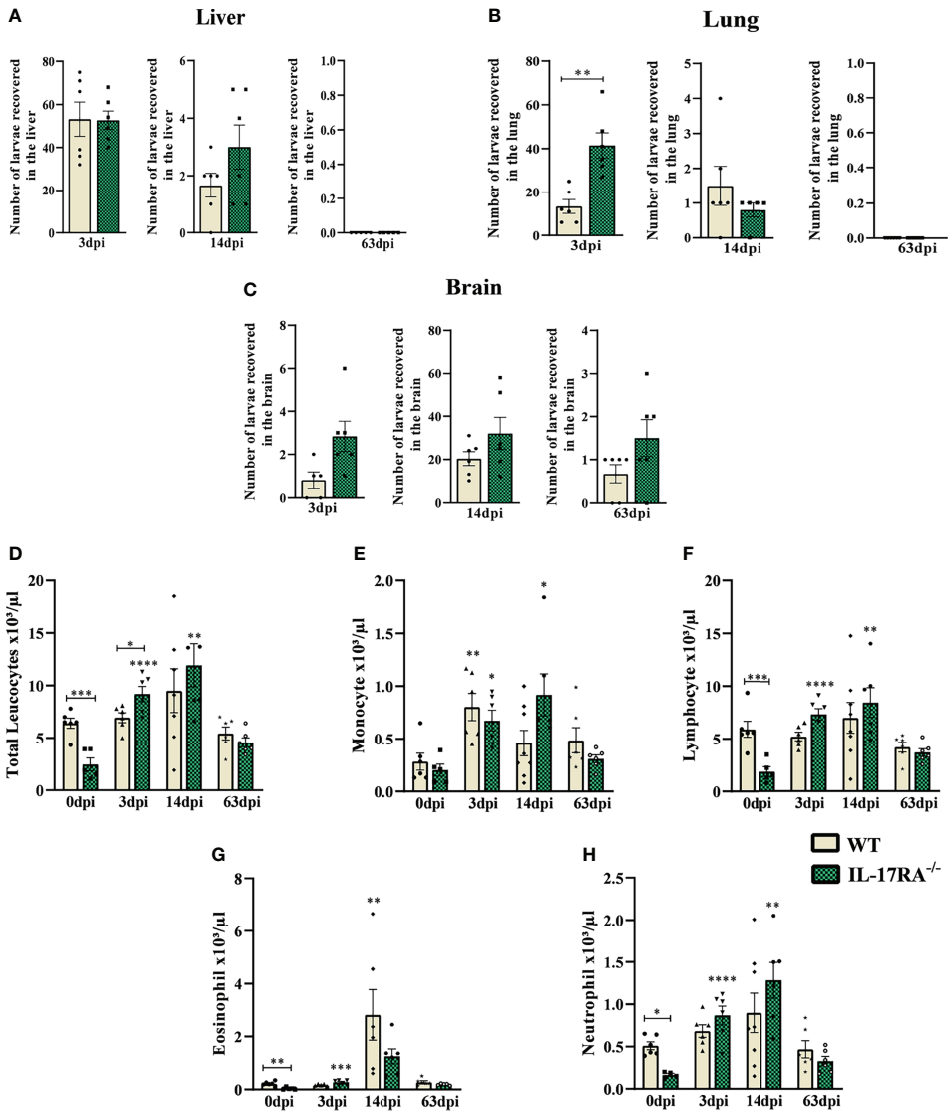

FIGURE 2
Parasite load and blood leukocyte count from WT and IL-17RA-/- mice during *T. canis* infection. Parasite burden was assessed by counting the total number of larvae in the liver, lung, and brain after 3, 14, and 63 dpi. Blood was collected by cardiac puncture for leukocyte count. **(A)** The number of larvae recovered from the liver in 3 days post-infection (dpi), 14dpi and 63dpi; **(B)** Number of larvae recovered from lung in 3dpi,14dpi and 63dpi; **(C)** Number of larvae recovered from the brain in 3dpi,14dpi and 63dpi; **(D)** Total leukocytes count; **(E)** Monocytes count; **(F)** Lymphocytes count; **(G)** Eosinophils count; **(H)** Neutrophils count. Statistical analyses were performed between each strain with its uninfected group (0dpi), represented by the asterisk without the bar, and between the two strains at the same time of infection, represented by the asterisk with the bar. Non-significant differences were not reported in the bar. Results represent the mean ± Standard error of the mean (SEM), Cream bars represent WT mice and green bars represent IL17RA-/- mice, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. According to the normality test, Student's t test was used in graphs A for 3dpi and 14dpi, B for 3dpi and C for 3dpi and 14dpi in the comparison between WT and IL-17RA-/- on each day of infection (dpi). The Mann-Whitney test was used in graphs A for 63dpi, B for 14dpi and 63dpi and C for 63dpi in comparing WT and IL-17RA-/- on each day of infection (dpi). One-way ANOVA test followed by Tukey's test was used on D, E, F, G, H graphs. N = 6 mice per experimental group.

To assess the systemic effects of *T. canis* infection, a differential blood cell count was performed in the acute and chronic phases of the infection (**Figures 2D–H**). The results showed that IL-17RA$^{-/-}$ mice naturally have fewer total leukocytes than WT ($p = 0.0008$), with lower numbers of eosinophils ($p = 0.0025$), neutrophils ($p = 0.0443$), and lymphocytes ($p = 0.0005$). However, there was an increase in leukocytes in IL-17RA$^{-/-}$ mice in the acute phase of infection (3dpi) ($p = 0.0486$), while leukocyte counts remained stable in WT mice (**Figure 2D**). At 3dpi, an increase in monocytes was observed in both strains, and this increase remained up to 14dpi in IL-17RA$^{-/-}$ mice compared to their control (**Figure 2E**). Also, in IL-17RA$^{-/-}$ mice, an increase in lymphocytes and neutrophils was observed at 3dpi and 14dpi (**Figures 2F, H**) compared to the uninfected. In WT mice, we observed the eosinophilia peak at 14dpi when compared to its control (**Figure 2G**).

T. canis infection induces increased TNF-α expression by non-classical monocytes regardless of the presence of IL-17RA receptor

Monocytes from peripheral blood were gated as LiveCD11b+CX3CR1+ and subdivided as classical (Ly6C^{high}) and non-classical (Ly6C^{low}) by multiparametric flow cytometry (**Supplementary Figures 1** and **2**). No difference was observed in the total monocyte population after infection in WT or IL-17RA$^{-/-}$ mice; however, when we analyzed the percentage of subpopulation of classical monocytes (Ly6C^{high}), we observed that uninfected WT 0dpi mice showed a higher percentage of Ly6C^{high} monocytes ($p = 0.0013$) and MHCII in Ly6C^{high} ($p = 0.0065$) compared to IL-17RA$^{-/-}$ (**Figures 3A–E**).

The results showed that infected WT mice increased the percentage of MHCII in non-classical monocytes compared to their uninfected group (WT 0dpi) and that infection by *T. canis*, regardless of the presence of IL-17RA$^{-/-}$, induced an increase in TNF-α expression in non-classical monocytes when compared to their respective uninfected groups.

The mean fluorescence intensity (MFI) shown by tSNE (**Figures 3E–H**), indicated that there is greater expression of MHCII in the WT 0dpi and IL-17RA$^{-/-}$ 3dpi groups, whereas the expression of TNF-α indicated to be greater in the WT 0dpi groups and in the infected groups.

The IL-17RA deficiency increases the frequency of eosinophils in peripheral blood during *T. canis* infection

Peripheral blood cytometry was performed to evaluate eosinophils (LiveSiglecF+) and neutrophils (LiveSiglecF-Ly6G+) (**Supplementary Figures 1** and **3**). Infection with *T. canis* led to increased frequency of both eosinophils and neutrophils in IL-17RA$^{-/-}$ mice compared to IL-17RA$^{-/-}$ 0dpi (**Figure 4A**). When comparing the two strains, a higher frequency of eosinophils was demonstrated in the IL-17RA$^{-/-}$ 3dpi groups compared to the WT 3dpi ($p = 0.0016$). After separating eosinophils and neutrophils and identifying their presence in all groups (**Figures 4B, C**), in the dimensional reduction analysis, 3 eosinophils (E1-E3) and 3 neutrophil clusters (N1-N3) were clustered (**Figures 4D, E**). When observing the eosinophils, we observed that the E1 that had the highest expression of MHCII was present in all groups, the E2 present mainly in the WT 0dpi group had the highest expression of CD69, MHCII and IL-4 and the E3 present mainly in the infected groups had the highest expression of IL-17, IL-4 TNF-α and CD69 (**Figure 4F**).

When we analyzed the neutrophil clusters, we observed that the N1 present in all groups had a higher expression of TNF-α, the N2 present mainly in the infected groups showed the highest expression of IL-17 and IL-4, while the N3 present in all groups except for WT 0dpi, showed low expression of all markers used. Thus, we can infer that *T. canis* infection can induce a higher frequency of eosinophils expressing IL-17, TNF-α, IL-4 and CD69 and neutrophils expressing IL-17 and IL-4.

It is important to mention that the clusters reflect only the patter of expression of the above stated markers and do not reflect different development stages of eosinophils or neutrophils.

In the acute phase of *T. canis* infection, no difference in the CD4 and CD8 T cell population was observed

To differentiate between CD4+ T lymphocyte and CD8+ T lymphocyte populations, we used the CD4 and CD8 markers (**Figure 5** and **Supplementary Figure 1**). When analyzing the frequencies of these populations, we did not observe statistical differences between the experimental groups (**Figures 5A–N**).

Regarding the markers and cytokines used for CD4 and CD8 lymphocytes, we observed that infected WT mice reduced the frequency of IL-4 compared to their control, while the IL-17RA$^{-/-}$ 0dpi mice naturally showed a reduction in the frequency of CD27 compared to WT 0dpi. We did not observe significant differences between the infected groups.

The IL-17RA receptor deficiency leads to increased IL-6 levels in the lungs after *T. canis* infection

For immunological analysis of the lung parenchyma in the acute phase of infection, cytokines from Th1, Th2, Th17, and Treg responses were measured by ELISA (**Figures 6A–K**). Infected WT mice showed an increase in Th2 cytokines (IL-5 and IL-33) compared to uninfected controls (0dpi). On the other hand, IL-17RA$^{-/-}$ mice showed an increase in IL-1ß, IL-4, IL-5, and IL-33. When comparing the two strains, it was observed that IL-17RA$^{-/-}$ mice increased the concentration of IL-6 after infection ($p = 0.0481$). Thus, these results demonstrate that in *T. canis* infection, the absence of the IL-17RA receptor favors an increase in IL-1ß, IL-4, and IL-6 in the lung parenchyma.

IL-17RA receptor deficiency increases the frequency of neutrophils expressing IL-4 in bronchoalveolar lavage during *T. canis* infection

BAL cytometry was performed to evaluate alveolar macrophages (LiveCD45+SiglecF+CD11c+), eosinophils (LiveCD45+CD11c-SiglecF+), neutrophils (LiveCD45+CD11c-SiglecF-Ly6G+) and CD4+ T lymphocytes (LiveCD45+low

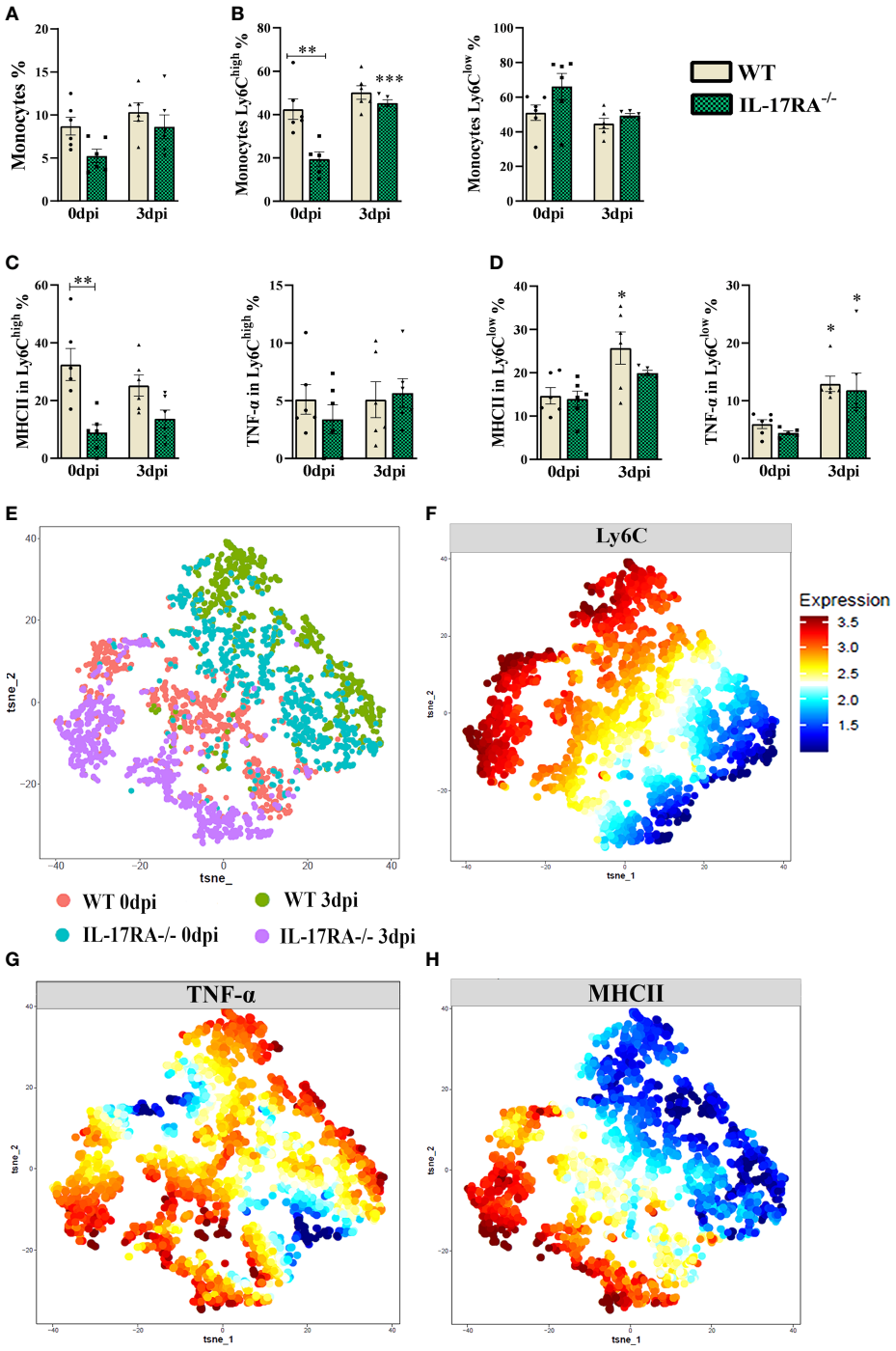

FIGURE 3
TNF-α and MHCII expression in Ly6C^{low} and Ly6C^{high} monocytes populations from WT and IL-17RA-/- mice during *T. canis* infection. **(A)** Bar show frequency of total monocytes; **(B)** Bar show frequency of classic monocytes Ly6C^{high} (left) and non-classical monocytes Ly6C^{low} (right); **(C)** Bar show frequency of MHC in classic monocytes Ly6C^{high} (left) and TNF-α classic monocytes Ly6C^{high} (right); **(D)** Bar show frequency of MHC in classic monocytes Ly6C^{low} (left) and TNF-α classic monocytes Ly6C^{low} (right); **(E)** Unsupervised high dimensional analysis of flow cytometry data (tSNE), gated on LiveCD11b+CX3CR1+ monocyte from combined data of blood obtained from WT 0dpi (red), WT 3dpi (green), IL17RA-/- 0dpi (blue) and IL17RA-/- 3dpi (purple); **(F)** Expression levels of Ly6C markers; **(G)** Expression map TNF-α; **(H)** Expression map MHCII. Statistical analyses were performed between each strain with its uninfected group (0dpi), represented by the asterisk without the bar, and between the two strains at the same time of infection, represented by the asterisk with the bar. Non-significant differences were not reported in the bar. Data represented as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001. For data that passed the normality test [**A**, **B** (left), **C** (right) and **D** (left)] One-way ANOVA test followed by the Tukey test was used, while non-parametric data B (right), C (left) and D (right) were used Kruskal-Wallis test followed by the Dunn test. N = 6 animals per group with two independent experiments.

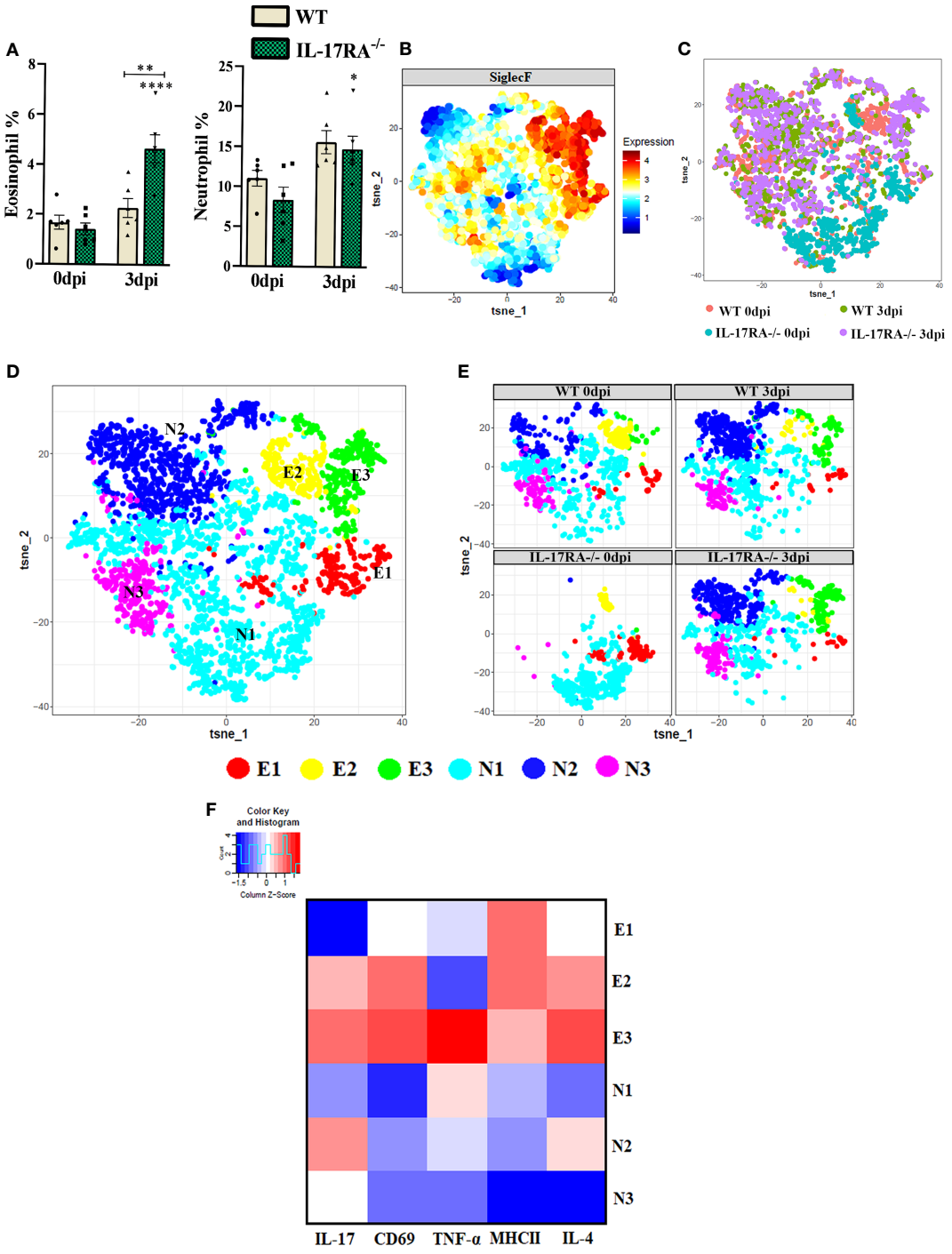

FIGURE 4
Analysis of neutrophil and eosinophil clusters of peripheral blood by cytometry in WT and IL-17RA-/- mice during *T. canis* infection. **(A)** Bar show frequency of total eosinophils (left) and neutrophils (right); **(B)** Expression levels of SiglecF; **(C)** Unsupervised high dimensional analysis of flow cytometry data (tSNE), gated on SiglecF+ eosinophils and SiglecF- and Ly6G+ neutrophils from combined data of blood obtained from WT 0dpi (red), WT 3dpi (green), IL17RA-/- 0dpi (blue) and IL17RA-/- 3dpi (purple); **(D)** tSNE of "C" representing cluster analysis by rPhenograph, eosinophils "E" and neutrophils "N"; **(E)** tSNE of "C" depicting neutrophil and eosinophil clusters per group; **(F)** Heat Map with cluster marker expression. Statistical analyses were performed between each strain with its uninfected group (0dpi), represented by the asterisk without the bar, and between the two strains at the same time of infection, represented by the asterisk with the bar. Non-significant differences were not reported in the bar. Data represented as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001. According to the normality test, One-way ANOVA test followed by the Tukey test was used. N = 6 animals per group with two independent experiments.

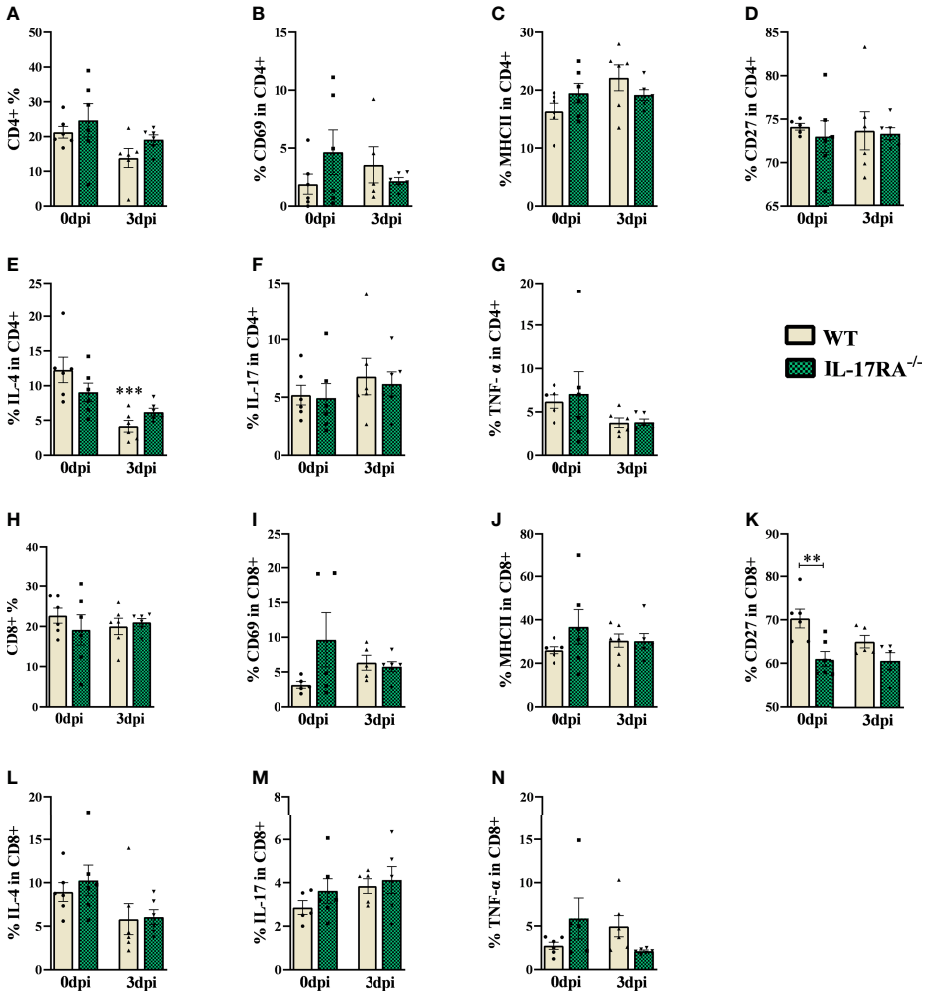

FIGURE 5
Flow cytometry analysis of TCD4 and TCD8 lymphocytes of peripheral blood in WT and IL-17RA-/- mice during *T. canis* infection. **(A)** Bar show frequency of total CD4 T lymphocytes; **(B)** Bar show frequency of CD69 in CD4 T lymphocytes; **(C)** Bar show frequency of MHCII in CD4 T lymphocytes; **(D)** Bar show frequency of CD27 in CD4 T lymphocytes; **(E)** Bar show frequency of IL-4 in CD4 T lymphocytes; **(F)** Bar show frequency of IL-17 in CD4 T lymphocytes; **(G)** Bar show frequency of TNF-α in CD4 T lymphocytes; **(H)** Bar show frequency of total CD8 T lymphocytes; **(I)** Bar show frequency of CD69 in CD8 T lymphocytes; **(J)** Bar show frequency of MHCII in CD8 T lymphocytes; **(K)** Bar show frequency of CD27 in CD8 T lymphocytes; **(L)** Bar show frequency of IL-4 in CD8 T lymphocytes; **(M)** Bar show frequency of IL-17 in CD8 T lymphocytes; **(N)** Bar show frequency of TNF-α in CD8 T lymphocytes. Statistical analyses were performed between each strain with its uninfected group (0dpi), represented by the asterisk without the bar, and between the two strains at the same time of infection, represented by the asterisk with the bar. Non-significant differences were not reported in the bar. Data represented as mean ± SEM, **p < 0.01, ***p < 0.001. According to the normality test, One-way ANOVA test followed by the Tukey test was used. N = 6 animals per group with two independent experiments.

granularityCD4+) (Figure 7). When analyzing the frequencies of cells in uninfected mice, we observed a reduction in eosinophils ($p = 0.0074$) and an increase in CD4+ T lymphocytes ($p = 0.0006$) in IL-17RA$^{-/-}$ mice compared to WT mice. In infected groups, we observed increased alveolar macrophages ($p = 0.0460$) and decreased neutrophils ($p = 0.0005$) in IL-17RA$^{-/-}$ mice compared to WT mice.

When we analyzed the expression of cytokines in alveolar macrophages, we observed that naturally IL-17RA$^{-/-}$ mice have a reduction in IL-10 compared to WT ($p = 0.0470$), and that during *T. canis* infection there is a reduction in IL-10 expression in these animals. Our results also showed that during *T. canis* infection there is an increase in IL-4 expression in these macrophages, being lower in IL-17RA$^{-/-}$ 3 dpi mice compared to WT 3dpi ($p = 0.0156$) (Figures 7A–D).

Dimensional reduction analysis was able to identify 1 CD4+ T lymphocyte cluster, 2 eosinophil clusters (E1 and E2) and 2 neutrophil clusters (N1 and N2) (Figures 7P, Q). When analyzing the frequency of CD4+ cells, it was observed that IL-17RA$^{-/-}$ 0dpi mice showed an increase in the frequency of CD4 T lymphocytes compared to WT 0dpi and that after infection there was a reduction in the frequency of these cells in the BAL in

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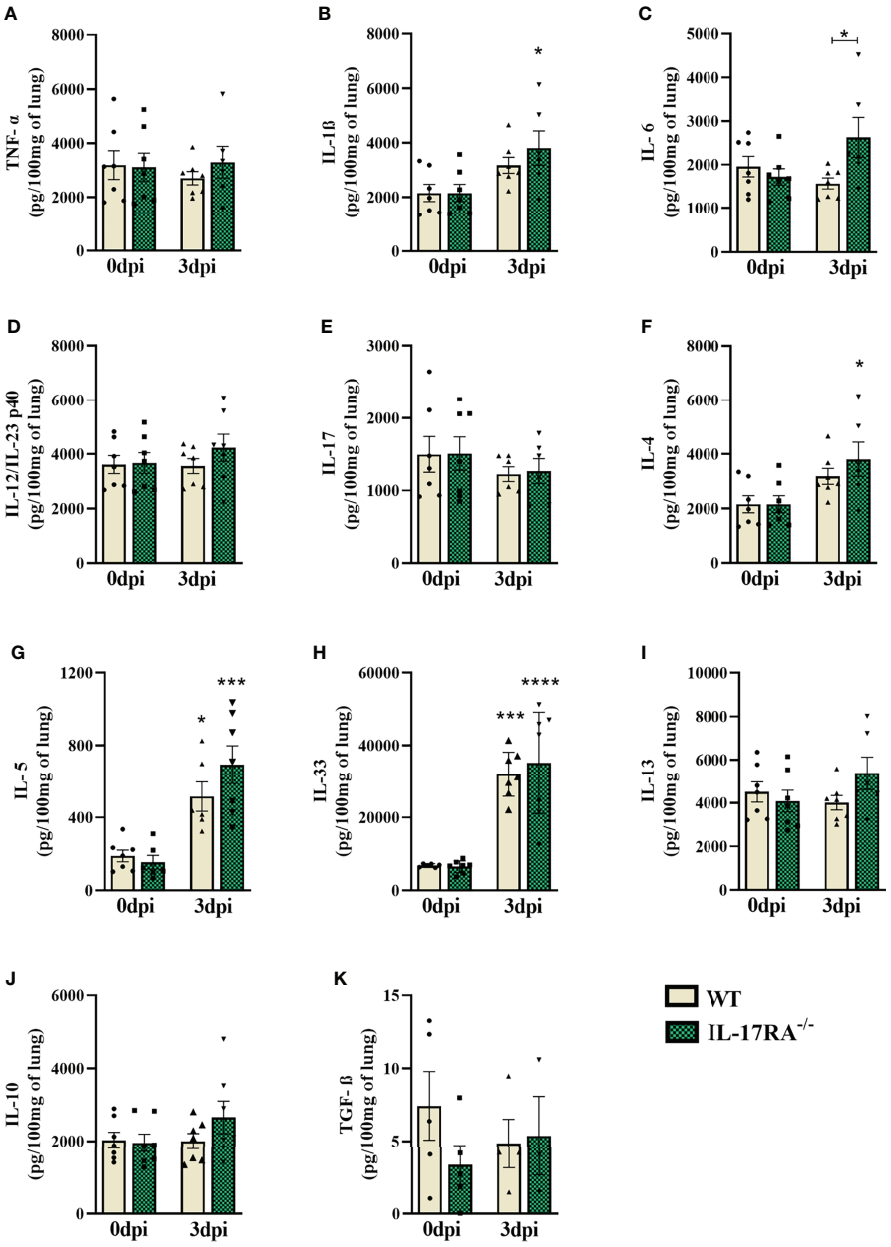

FIGURE 6
Cytokine profile in the lung parenchyma of WT and IL-17RA-/- mice during *T. canis* infection. The cytokines present in the lung parenchyma were measured by ELISA. **(A)** TNF-α; (B) IL-1β; (C) IL-6; **(D)** IL-12/IL-23p40; **(E)** IL-17; **(F)** IL-4; **(G)** IL-5; **(H)** IL-33; **(I)** IL-13; **(J)** IL-10; **(K)** TGF-β. Statistical analyses were performed between each strain with its uninfected group (0dpi), represented by the asterisk without the bar, and between the two strains at the same time of infection, represented by the asterisk with the bar. Non-significant differences were not reported in the bar. Results represent the mean ± SEM, Cream bars represent WT mice and green bars represent IL17RA-/- mice, *p < 0.05, ***p < 0.001, ****p < 0.0001. According to the normality test, One-way ANOVA test followed by Tukey test was used. N = 7 mice per experimental group.

IL-17RA$^{-/-}$ mice. It was also possible to observe an increase in the expression of IL-17 in these cells in WT 3dpi mice compared to their respective control group (**Figures 7E, F**).

Regarding eosinophils, we observed an increase in IL-10 in WT 3dpi and IL-4 mice in both infected groups compared to their respective control. We also observed a reduction in the MFI of MHCII in IL-17RA$^{-/-}$ 3dpi mice compared to IL-17RA$^{-/-}$ 0dpi (**Figures 7G–J**). In the dimensional reduction analysis, we observed that the E1 cluster is present in all groups except for WT 0dpi, while E2, although present in all groups, stands out in the uninfected groups (**Figures 7P, Q**).

In neutrophils, we observed a reduction in MHCII and IL-10 in the infected groups compared to their respective control groups. We also observed an increase in IL-4 in these cells in

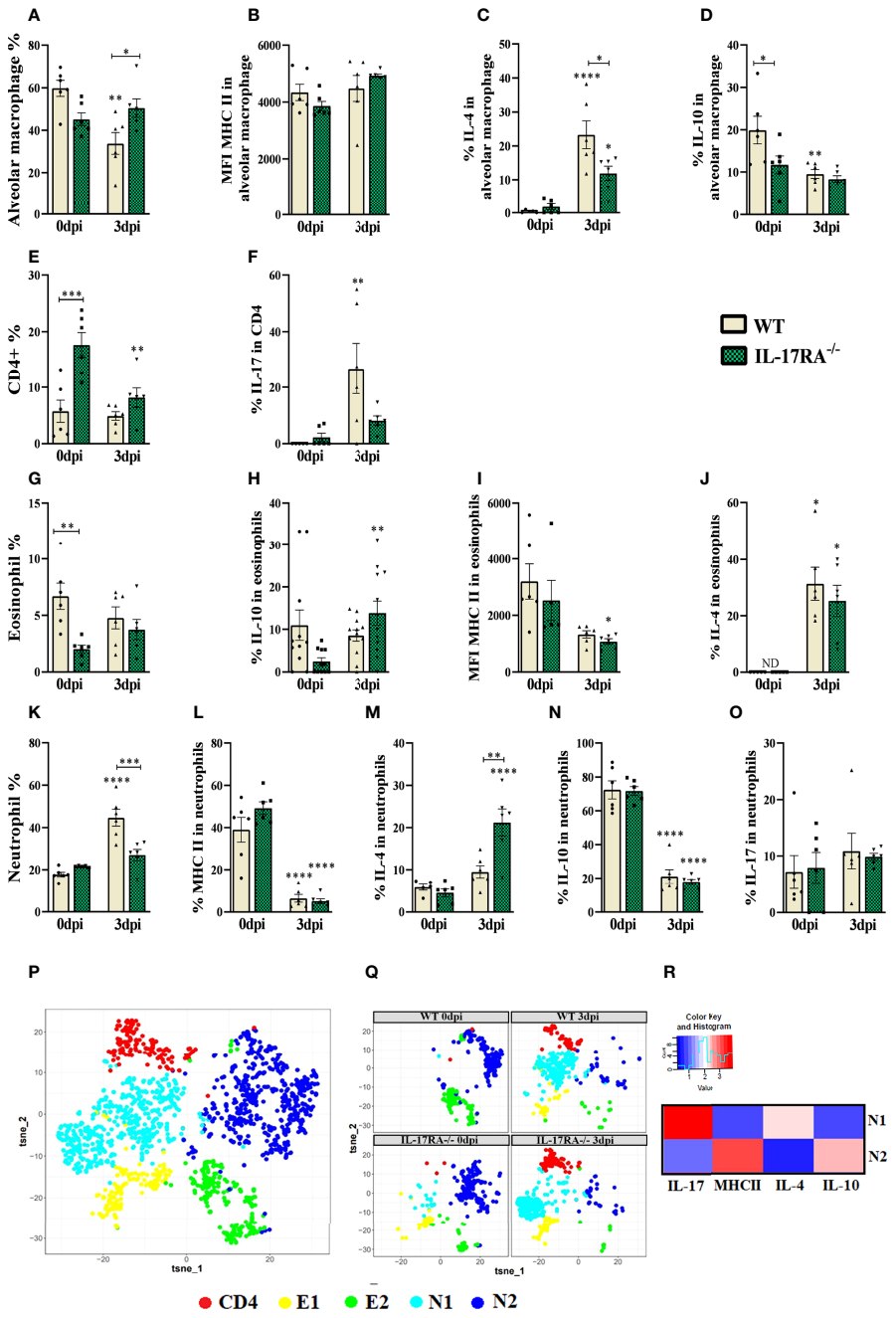

FIGURE 7
Flow Cytometry of bronchoalveolar lavage (BAL) analysis of leukocyte populations, alveolar macrophages, eosinophils, CD4+ and neutrophils in WT and IL-17RA-/- mice during *T. canis* infection. **(A)** The bar shows the frequency of alveolar macrophage; **(B)** The bar shows the mean intensity of fluorescence (MFI) of MHCII in alveolar macrophage; **(C)** The bar shows the frequency of IL-4 in alveolar macrophage; **(D)** The bar shows the frequency of IL-10 in alveolar macrophage; **(E)** The bar shows the frequency of CD4 T lymphocytes; **(F)** The bar shows the frequency of IL-17 in CD4 T lymphocytes; **(G)** The bar shows the frequency of eosinophils; **(H)** The bar shows the frequency of IL-10 in eosinophils; **(I)** The bar shows the MFI of MHCII in eosinophils; **(J)** The bar shows the frequency of IL-4 in eosinophils. ND= not-detected; **(K)** The bar shows the frequency of neutrophils; **(L)** The bar shows the frequency of MHCII in neutrophils; **(M)** The bar shows the frequency of IL-4 in neutrophils; **(N)** The bar shows the frequency of IL-10 in neutrophils; **(O)** The bar shows the frequency of IL-17 in neutrophils; **(P)** tSNE representing cluster analysis by rPhenograph, eosinophils "E", neutrophils "N" and CD4 T lymphocytes "CD4"; **(Q)** tSNE of "P" depicting eosinophils, neutrophils and CD4 T lymphocytes clusters per group; **(R)** Heat Map with neutrophils cluster marker expression. Statistical analyses were performed between each strain with its uninfected group (0dpi), represented by the asterisk without the bar, and between the two strains at the same time of infection, represented by the asterisk with the bar. Non-significant differences were not reported in the bar. Data represented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. For data that passed the normality test **(A–E, G, K–N)** One-way ANOVA test followed by the Tukey test was used, while non-parametric data **(F, H, I, J, O)** were used Kruskal-Wallis test followed by the Dunn test. N = 6 animals per group with two independent experiments.

IL-17RA$^{-/-}$ 3dpi mice compared to WT 3dpi ($p = 0.0014$). When analyzing the neutrophil populations by dimensional reduction, we observed that the population of N1 neutrophils is found in greater quantity in the infected groups, and they express IL-17 and IL-4, while the N2 population is found in the uninfected groups, and these express IL-10 and MHCII (**Figures 7K–R**).

Thus, we observed that *T. canis* infection induces an increase in IL-17-producing CD4+ T lymphocytes and IL-4 and IL-10-producing eosinophils, and the absence of IL-17RA increases the frequency of IL-4-producing neutrophils and reduces the frequency of alveolar macrophages expressing the same cytokine.

IL-17RA receptor signaling contributes to the formation of lung inflammatory nodular aggregates during the *T. canis* infection

When analyzing the protein and hemoglobin levels in the BAL, the IL-17RA$^{-/-}$ 3dpi mice had a higher protein concentration than the WT 3dpi ($p = 0.0057$), probably due to the higher parasite load and higher levels of higher cytokine-producing cells regulators (**Figures 8A, B**). In the histopathological analysis, we did not observe significant differences in the inflammation and hemorrhage scores; however, the IL-17RA$^{-/-}$ 3dpi mice reduced the number of inflammatory nodular aggregates ($p < 0.0001$) compared to WT mice 3dpi (**Figures 8C–H**). We also analyzed the activity of N-acetylglucosaminidase (NAG), eosinophilic peroxidase (EPO) and neutrophil myeloperoxidase (MPO) in lung tissue, with no significant difference between the infected groups (**Supplementary Figure 5**).

The histopathological analysis of the lung parenchyma of mice belonging to the group's WT 0dpi, IL-17RA$^{-/-}$ 0dpi, WT 3dpi, IL-17RA$^{-/-}$ 3dpi infected with *T. canis* was performed. It was possible to observe and describe the lesions of the lung parenchyma caused by larval migration of the parasite in terms of topography, inflammatory infiltrate, presence or absence of larvae, inflammatory nodular aggregates, vascular and exudative phenomena (**Figure 8I**).

Regarding mice belonging to the WT 3dpi group, when the histopathological analysis was performed, thickening of the interalveolar septa was observed at the expense of the mixed inflammatory infiltrate characterized by eosinophils and neutrophils, macrophages and, in a smaller amount, constituted by lymphocytes. Multifocal nodular aggregates constituted predominantly by eosinophils and to a lesser extent by neutrophils, macrophages, and lymphocytes were frequently observed in all mice, often located around larval fragments. Exudative phenomena such as perivascular edema and hemorrhagic areas were also evidenced. Hypertrophy of the epithelial cells of the bronchi and bronchioles has often been observed. Most mice had larvae dispersed in the lung parenchyma and often close to the hemorrhagic zones.

When evaluating the lung parenchyma of mice belonging to the IL-17RA$^{-/-}$ 3dpi group, an increase in the thickening of the interalveolar septa was observed at the expense of the predominantly mononuclear inflammatory infiltrate characterized by macrophages and lymphocytes, and sometimes neutrophils and eosinophils were found. Sometimes, in some mice, the presence of multifocal nodular aggregates constituted predominantly by eosinophils and, to a lesser extent, by neutrophils, macrophages, and lymphocytes were observed. The observed nodular aggregates were smaller and smaller when compared to those observed in the WT 3dpi group. Exudative phenomena such as perivascular edema, large hemorrhagic areas, and vascular congestion were frequently evidenced, contributing to the thick appearance of the alveolar septa. Hypertrophy and hyperplasia of the bronchial epithelial cells were also frequently observed. In all mice, larvae were found scattered in the lung parenchyma and often close to the hemorrhagic zones. The histopathological analysis of the lung parenchyma of mice belonging to the control groups WT 0dpi, IL-17RA$^{-/-}$ 0dpi exhibited a regular morphological aspect, without histopathological alterations.

Discussion

In toxocariasis, the immune response triggered during infection is associated with eosinophilia in peripheral blood, eosinophilic infiltration around larval migration sites, and a production of type 2 (Th2) T helper immune response (20). Recent studies demonstrate that several cytokines are present during the acute and chronic phases of the disease, and among them is IL-17, which is increased during infection (13, 14). In this way, the study of the role of the IL-17 pathway in toxocariasis becomes essential to expand the immunological knowledge about the disease and provide avenues for the development of drugs and therapeutic targets that can prevent larval migration in accidental hosts. For the study of IL-17A/IL-17RA axis, mice genetically deficient for IL-17RA generated from C57BL/6 mice were used (21).

Upon penetrating the intestinal mucosa in incidental hosts, *T. canis* larvae migrate mainly towards the liver, lungs, and brain. They may also travel to other organs (e.g., skeletal muscle, heart, and eyes) by mechanical means and digestion by protease (16). The presence of the larva triggers an acute inflammatory reaction, resulting mainly from innate immunity to *T. canis* excretion-secretion (TES) antigens (22). In our study, we observed an increase in the parasite load in the lungs of IL-17RA mice at 3dpi. In a previous study, a change in larval load was also observed with 3dpi in the lungs in GATA1$^{-/-}$ mice infected with *T. canis* compared to WT (23). Although parasite migration is erratic, at 3dpi is the peak of larval migration in the lung (13), thus we found a greater number of larvae at this time of infection, which may have shown greater statistical differences between the groups.

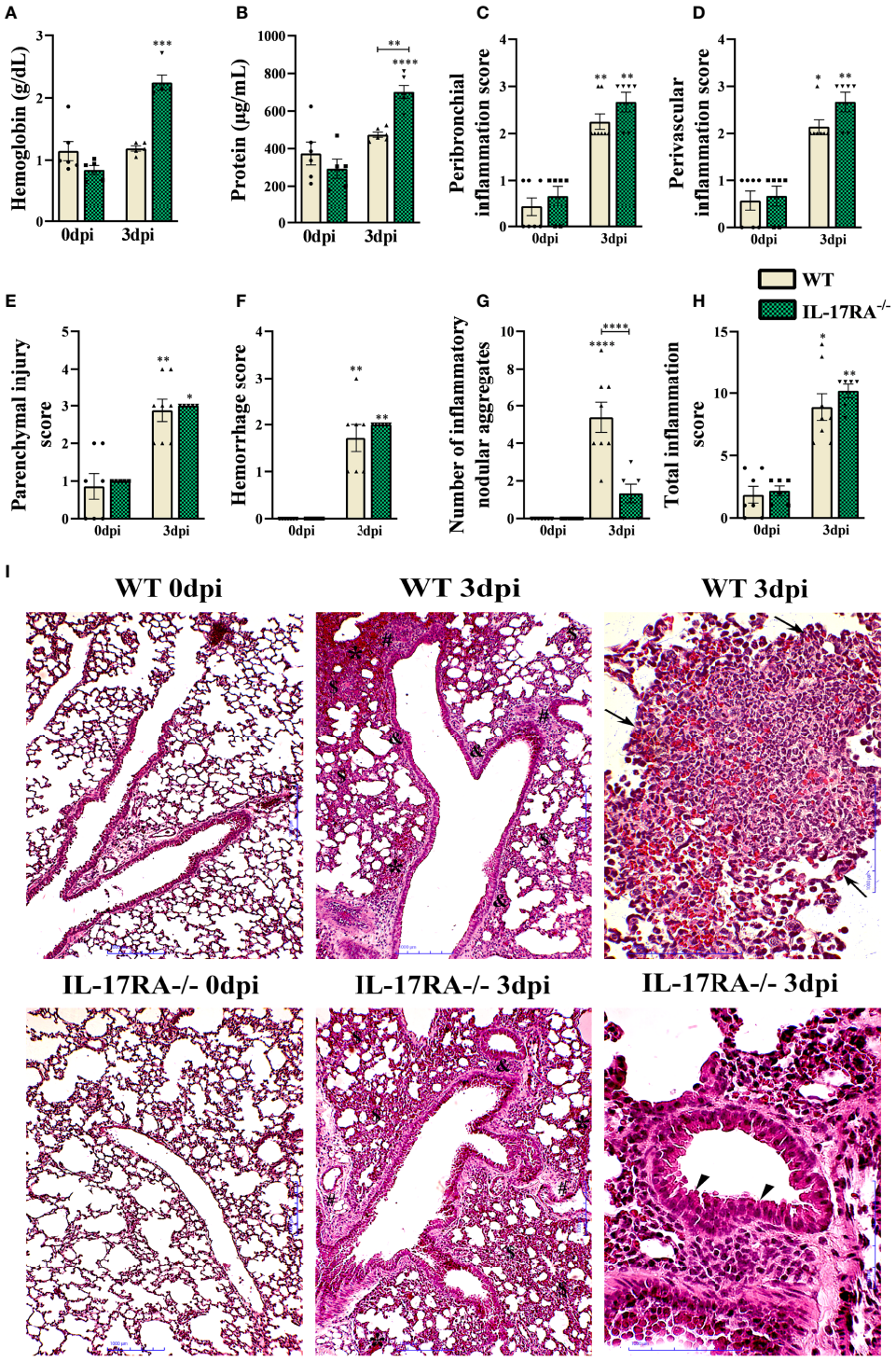

FIGURE 8
Characterization of lung parenchyma injury and inflammation in WT and IL-17RA-/- mice during *T. canis* infection. **(A)** Hemoglobin quantification in the bronchoalveolar lavage (BAL); **(B)** Total protein levels in BAL; **(C)** Peribronchial inflammation score; **(D)** Score for perivascular inflammation; **(E)** Parenchymal injury score; **(F)** Bleeding score; **(G)** Number of inflammatory nodular aggregate; **(H)** Total inflammation score; **(I)** Histopathology of lung tissue. Representative hematoxylin and eosin staining of lung sections. Bronchial epithelial hypertrophy and hyperplasia (arrowhead), inflammatory nodular aggregate (arrows), bleeding area (*), parenchymal inflammation ($), airway inflammation (&), vascular inflammation (#). Bar = 1000µm. Statistical analyses were performed between each strain with its uninfected group (0dpi), represented by the asterisk without the bar, and between the two strains at the same time of infection, represented by the asterisk with the bar. Non-significant differences were not reported in the bar. Data represented as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. For data that passed the normality test, B, One-way ANOVA test followed by the Tukey test was used, while non-parametric data **(A, C, D, E–H)** were used Kruskal-Wallis test followed by the Dunn test. N = 7 mice per experimental group.

Although there is scarce data related to IL-17/IL-17RA axis in toxocariasis in the literature, studies relating IL-17 to helminth infections have expanded. Nogueira et al. (9) observed that mice, after multiple exposures to *Ascaris suum*, exhibited greater control of larval migration due to intense lung inflammation associated with a systemic Th2/Th17 immune response. In murine schistosomiasis, the absence of IL-17A signaling has been related to reduced liver fibrosis (6). Resende et al. (13) demonstrated that in toxocariasis, there is a mixture of Th2 and Th17 inflammatory responses, observed by the increase of the cytokines IL-4, IL-5, IL-13, IL-6, and IL-17 in the serum of mice, during the phase of larval migration, showing that *T. canis* larvae are capable of triggering IL-17 production. Previous studies by our group also observed that the IL-33/ST2 pathway in mice infected with *T. canis* increased hepatic and brain parasite load and reduced IL-17 activity (14). In our study, the presence of IL-17RA appears to be relevant to reduce the parasite load in the acute phase of infection in the lungs, and this control in the initial phase is essential to prevent a greater number of larvae from migrating to more susceptible organs, such as the brain.

In *T. canis* infection, leukocytosis is expected, with neutrophil recruitment followed by eosinophil activation, which is usually increased during the acute inflammation phase but may persist until the chronic phase of infection (24). In our study, leukocytosis was observed only in IL-17RA$^{-/-}$ 3dpi mice. The cytokine IL-17 is important for neutrophil biology, also is a potent stimulator of lung microvascular endothelial cells to produce the neutrophil chemoattractant (CXCL8 and derivatives of the 5-lipoxygenase pathway) that selectively recruits these cells to sites of inflammation (25). Therefore, the absence of leukocytosis in WT mice is due to the targeting of immune cells to the tissues, especially the lungs, as shown in our study.

Classical monocytes are essential for initiating the inflammatory response, while non-classical monocytes have been widely viewed as an anti-inflammatory, maintaining vascular homeostasis. When analyzing the cells present in the blood of WT and IL-17RA$^{-/-}$ mice, we observed the presence of classic and non-classical monocytes, and in the mice infected by *T. canis*, these cells showed higher expression of TNF-α. TNF-α appears to play a crucial role in regulating the survival and function of monocytes in the periphery (26, 27). In the serum of patients infected with *T. canis*, an increase in TNF-α and IL-10 was observed (28). On the other hand, Resende et al. (13) did not observe significant changes in the levels of IL-10, TNF-α, or IFN-γ in mice infected with *T. canis*. Also, in the peripheral blood of mice infected with *T. canis*, an increase in the cytokines IL-4, IL-5 and IL-10 was observed, but not in TNF-α (20).

As we depicted by the analysis of blood eosinophils using the flow cytometry, the IL-17RA$^{-/-}$ mice showed increased frequencies of eosinophils during *T. canis* infection compared to WT mice. In response to damage or some types of infections, eosinophils are recruited to sites of inflammation where they secrete cytokines such as IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL- 16, IL-18, TGF, toxic granular proteins, lipid mediators, in addition to being capable of inducing tissue damage and dysfunction (29). It has also been shown that eosinophils constitutively express receptors for IL-17A and IL-17F, and it is hypothesized that their Th17-mediated activation could induce the release of pro-inflammatory cytokines and chemokines (30).

Although classically described the importance of CD4+ and CD8+ T lymphocytes, γδ T lymphocytes and NK cells in the expression of IL-17, studies have shown that eosinophils and neutrophils can produce large amounts of IL-17 in the lung, which is related to inflammation lung disease in allergic models and in infections (2, 31-36). Moreover, eosinophil-derived IL-17 contributes to lung tissue injury and inflammation, controlling the expansion of IL-17+ Th17 and Tγδ lymphocytes during *Aspergillus fumigatus* infection in mice (2). Thus, in the absence of the IL-17A receptor, eosinophils seem to be recruited to amplify the IL-17A response and, consequently, tissue inflammation. Neutrophils are the first cells of innate immunity to reach the injury site. In the present study, they were shown to be producers of IL-17 and IL-4, mainly in infected mice. Studies have reported the role of neutrophils as IL-17 producers in psoriasis, autoimmune diseases, bacterial infections, and helminth infections (37–39). For example, in Nippostrongylus brasiliensis infection, an alternatively activated (N2) neutrophil phenotype leads to developing a long-lasting M2 macrophage phenotype, which subsequently mediates parasitic larval damage (40). Rodolpho et al. (41) demonstrated that in BALB/c mice during *T. canis* infection, peripheral blood eosinophils show an upregulated expression of activation markers such as CD69, MHCII, CD80, CD86, as demonstrated in our study.

CD27 is a co-stimulation molecule expressed on naive CD4+ and TCD8+ T lymphocytes and, unlike other members of the TNF receptor family, is released from the cell surface after T cell activation (42). CD69 is a marker expressed on the surface of T lymphocytes after the involvement of the T cell receptor (TCR) with CD3. This marker activates cytokines, performs mitogenic polyclonal stimulation, carries out the targeting and migration of lymphocytes, and appears to be an early controller of Th17 differentiation, preventing the differentiation of T cells towards Th17 (43, 44). In our study, we did not observe differences in the activation markers CD27, MHCII and CD69. Our results were probably due to the evaluation of the infection time, which was at 3dpi, not being sufficient to assess the differentiation and activation of lymphocytes.

During migration, *T. canis* larvae cause tissue damage and provoke inflammatory reactions, with the involvement of neutrophils, eosinophils, and lymphocytes in the infiltrate of the lungs of infected mice (45). Our study observed an increase in IL-6 cytokine in IL-17RA$^{-/-}$ mice compared to WT. The IL-17RA receptor is expressed in the lungs on lung fibroblasts, lung endothelial cells, and airway smooth muscle cells; signaling through this receptor induces the production of chemokines and the cytokine IL-6 (46).

In our study, we observed a reduction in the percentage of neutrophils and an increase in macrophages in the BAL of IL-17RA$^{-/-}$ infected mice. The IL-17 pathway is essential in the recruitment and activation of macrophages and especially neutrophils to fight extracellular bacteria and fungi on mucosal surfaces. Contributes to epithelial homeostasis in the skin and stimulates B cells, acting as a bridge between the innate and acquired immune system (36). Our data also showed that IL-4 and IL-17 seem to be important in the response to *T. canis* infection, produced by various BAL cells, such as macrophages, eosinophils and mainly by neutrophils, as demonstrated by the cytometry technique. Neutrophils express and produce cytokines constitutively or upon activation by microenvironmental stimulus and can be a significant source of pro-inflammatory and immunoregulatory cytokines. The main neutrophil secreted cytokines are IL-17 and IFN-γ; however, studies have also reported the production of IL-4, IL-10, and TGF-β (47). Our study indicated that neutrophils that express IL-17 are more present in mice infected with *T. canis*, suggesting that the microenvironment generated by the infection favors the production of this cytokine.

Alveolar macrophages are the most abundant innate immune cells in the distal lung, located on the luminal surface of the alveolar space and due to exposure to the high partial pressure of oxygen, surfactant, and signals provided by alveolar type I and II cells displayed a phenotype distinct from other cells, which allows them to be differentiated from transient monocyte-derived cells recruited into the alveolar space during tissue injury. These macrophages are identified by the expression of high levels of CD11c integrin and the lectin Siglec F (48). M2 macrophages are primarily activated by IL-4/IL-13 and IL-10 in response to injury, and they act to promote wound healing by attenuating inflammation and stimulating extracellular matrix formation (49, 50). Studies have shown that depending on the stimulus and if there are macrophages, they can secrete Th2 cytokines such as IL-4 and IL-13 (51, 52). Opsonized schistosome eggs antigens (SEA) have been shown to upregulate IL-4 production by C57BL/6 macrophages (52), as demonstrated in our study, in which there was an increase in the percentage of IL-4 in macrophages in WT 3dpi mice. In our study, we observed that the absence of the IL-17A receptor caused these macrophages to decrease the production of IL-4, we believe that the absence of IL-17RA changes the cytokine microenvironment to which macrophages are exposed, and in this way may even interfere on the type of differentiation in classical or alternatively activated macrophages.

In our study, we observed that several cells are sources of IL-17A and although the effect of these cytokines is more related to the induction of inflammation, IL-17 is not as potent in isolation. Its primary function is to recruit immune cells and act synergistically with other cytokines such as TNF, IL-1β, IFN-γ, GM-CSF and IL-22 (5, 53). Studies have indicated that there is a close link between IL-17A and the Th2 response, and it has been observed that in the initial phase of infection by *N. brasiliensis* there is a reduction in the levels of IFN-γ mediated by IL-17A, allowing the subsequent development of immunity type 2 in the lungs (12). However, while there is much evidence that effector cytokines on the IL-17/IL-17RA axis may play protective roles against infectious agents in the lung, there is increasing evidence that this pathway can also result in lung pathology (46). Thus, we believe that in the initial phase of infection by *T. canis*, the cytokines IL-17A and IL-4 are the main active components, however, a synergistic action with other components of the immune system is necessary for a more effective and controlled response.

Pulmonary infections by *T. canis* are characterized by pulmonary inflammation with cell aggregates, the presence of hemoglobin and protein extravasation, which alter vascular permeability, airway hyperresponsiveness, and, frequently, the presence of granulomas are observed (14, 45, 54, 55). In a previous study, BALB/c mice infected with *T. canis* with 3dpi showed thickening of the interalveolar septa with a mixed inflammatory infiltrate in the lungs, characterized by eosinophils, neutrophils, macrophages, and lymphocytes. The mice presented exudative phenomena, such as perivascular edema and extensive hemorrhagic areas, in addition to the presence of granulomas in the exudative phase, composed mainly of eosinophils and macrophages followed by lymphocytes (14). The infected mice in our study showed similar results; however, the IL-17RA$^{-/-}$ 3dpi mice showed an increase in hemoglobin and inflammatory nodular aggregates formed mainly by eosinophils, which later gave rise to granulomas. IL-17A plays a significant role in granuloma maturation, from early to mature stages, and is indispensable for the protective response against *Mycobacterium tuberculosis* infection in the lung (56). Paracoccidioidomycosis also showed that IL-6, IL-23, or IL-17RA receptor deficiency impaired granuloma formation and conferred susceptibility during infection (57). Thus, we demonstrate that the presence of the IL-17RA receptor is essential for the formation of inflammatory nodular aggregates, amplifying the inflammatory process that can generate greater tissue damage; however, as it is in the initial phase of infection, lung damage was not observed in these mice.

Taken together, this study demonstrates that the IL-17/IL-17RA axis contributes to reducing parasite burden but increases tissue inflammation during the acute phase of *T. canis* infection. Furthermore, the production of the cytokines IL-17A and IL-4, originating mainly from innate immune cells, seems to be important for controlling the larval lung burden at the beginning of the infection (**Figure 9**). However, the IL-17RA pathway triggers the formation of pulmonary nodular aggregates that suggest a future formation of granulomas that amplify the lung injury. In conclusion, our results suggest that, in the context of toxocariasis, the IL-17RA receptor may represent a promising therapeutic target to reduce organ inflammation and morbidity triggered by uncontrolled parasite migration, causing neurotoxocariasis and ocular toxocariasis. Finally, future studies are needed to reveal the mechanisms of pharmacological

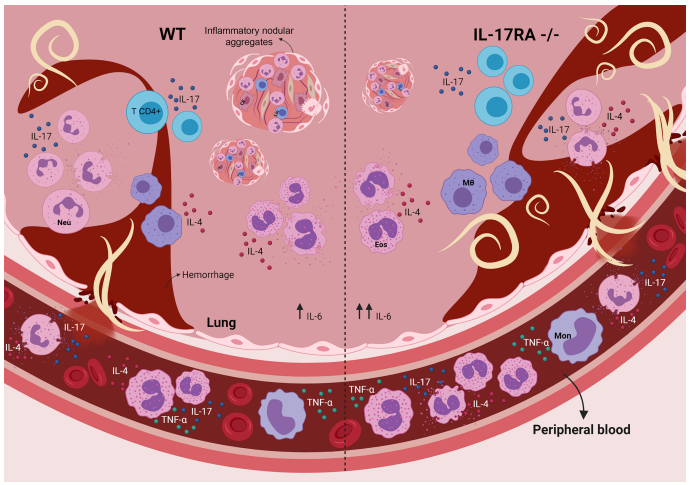

FIGURE 9
Role of the IL-17RA receptor in the context of T. canis infection in mice. The absence of the IL-17RA receptor during T. canis infection increases the frequency of eosinophils in the blood, contributes to the increase in the pulmonary parasite load, IL-6 production and the frequency of IL-4-secreting neutrophils and reduces the number of inflammatory nodular aggregators. MΦ, alveolar macrophage; CE, endothelial cell; Eos, eosinophil; Neu, neutrophil; Mon, monocyte; Lin, lymphocyte.

modulation of IL-17A during the parasite-host relationship in the context of toxocariasis.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

Ethics statement

The maintenance and use of mice were carried out following the recommendations of the Brazilian College of Animal Experimentation (COBEA). The present study was reviewed and approved by the Ethics Committee for Animal Experimentation (CEUA) of the Federal University of Minas Gerais, Brazil, through protocol #56/2018. All efforts were made to minimize animal suffering.

Author contributions

Conceived and designed the experiments: TL-S, CL, FV-S, FO, RF, LM, LB. Performed the experiments: TL-S, CL, FV-S, FO, LK, LP, CA, JS, LM. Analyzed the data: TL-S, FO, RR, LM, LB. Contributed reagents, materials, analysis tools: TL-S, FO, RR, RF, LM, LB. Wrote and reviewed the paper: TL-S, CL, FV-S, FO, LK, LP, CA, JN, RR, RF, LM, LB. All authors contributed to the article and approved the submitted version.

Funding

This investigation received partial support from Fundação de Amparo a Pesquisa do Estado de Minas Gerais/FAPEMIG, Brazil (Grant# CBB APQ-00766-18), the Brazilian National Research Council (CNPq) (Grant# 421392/2018-5 and Grant# 302491/2017-1) and Pró-Reitoria de Pesquisa of Universidade Federal de Minas Gerais to cover research inputs. TL-S is grateful for the Ph.D. fellowship provided by the Brazilian National Research Council (CNPq), Post-graduation Program in Infectology and Tropical Medicine/Universidade Federal de Minas Gerais. RR, RF, and LB are Research Fellows from the Brazilian National Research Council (CNPq). The funders had no role in study design, data collection, analysis, decision to publish, publication fees, or manuscript preparation.

Acknowledgments

We would like to thank Michele Silva de Matos and Vanessa Gomes Fraga for their technical assistance and support during the experiments. Figures were made using biorender.com.

Conflict of interest

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

The handling editor declared a past co-authorship with the authors TL-S, RF, LM, and LB.

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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.2022.864632/full#supplementary-material.

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frontiers | Frontiers in Immunology

OPINION
published: 01 July 2022
doi: 10.3389/fimmu.2022.933490

Opinion Article: NK Cells in Cutaneous Leishmaniasis: Protection or Damage?

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Keywords: natural killer, cutaneous leishmaniasis, immune response, tegumentary leishmaniasis, leishmania

OPEN ACCESS

Edited by:
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Reviewed by:
Tiago Rodrigues Ferreira, National Institute of Allergy and Infectious Diseases (NIH), United States

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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *01 May 2022*
Accepted: *01 June 2022*
Published: *01 July 2022*

Citation:
Cavalcante MKA, de Freitas e Silva R, Pereira VRA and Brelaz-de-Castro MCA (2022) Opinion Article: NK Cells in Cutaneous Leishmaniasis: Protection or Damage? Front. Immunol. 13:933490. doi: 10.3389/fimmu.2022.933490

INTRODUCTION: CL AND THE EARLY IMMUNE RESPONSE

Cutaneous Leishmaniasis (CL) is considered a neglected disease, mainly linked to low socioeconomic conditions (1, 2). Currently, estimates suggest 900,000 to 1.5 million new cases per year with 95% of the cases in the Americas, Mediterranean and Asia (3).

The disease clinical manifestation can range from a single to multiple lesions, being observed in 90% of cases a nodular ulcerative squamous lesion. After inoculation of the metacyclic promastigote of *Leishmania* into the host skin by the sandfly bite, a papule forms at the site of bite, and later it turns into an ulcerated lesion with delineated borders, a reddish background, and an intense inflammatory infiltration (lymphocytes, phagocytes, and plasma cells) (4–9).. Factors such as the species of *Leishmania* involved in the infection and the host immune response directly influence the lesion type and clinical outcome in the patient (10, 11).

After dermis inoculation the promastigotes cause activation of the complement system and factor C3b deposition on the parasite surface. However it has been demonstrated that *Leishmania* protease GP63 is able to inactivate C3b (12). These promastigotes are opsonized, by the C3 molecule of the complement system, to be phagocytized by phagocytic immune cells. Among these phagocytic cells are neutrophils, dendritic cells, and macrophages (13–15).

Neutrophils are the first cells to arrive at the infection site, being attracted by complement proteins, cytokines (e.g., IL-8), and chemokines (e.g., CXCL1 and CXCL2). These cells can eliminate the parasites through the action of nitric oxide (NO) and other reactive oxygen species (ROS), and also produce high levels of chemokines such as CXCL8 and CXCL9, responsible for the recruitment of more neutrophils and Th1 cells (16–18). In addition, because they are short-lived phagocytic cells, they promote the entry of more promastigotes in macrophages phagocyting dead neutrophils. Thus, neutrophils serve as a "trojan horses", but the macrophages also produce cytokines which will in turn activate other immune cells (19, 20).

Resident dendritic cells (DCs) seems to play a key role in the immunopathogenesis of CL (21, 22). Once these cells interact with *Leishmania* there is an increase in the expression of co-stimulatory molecules, such as CD40, CD80 and CD86 which are essential for T cell activation (23, 24). Thus, it is hypothesized that resident DCs recruit monocytes, which differentiate into monocyte-derived dendritic cells (moDCs). These cells have an intense phagocytic activity, and evidences suggests that they favor the growth and survival of parasites (25, 26).

When *Leishmania* spp. are phagocytized, they are trapped in vesicles called phagosomes, which then fuse with lysosomes and turn into acidic phagolysosomes. From this point on, cellular processes such as oxidative stress and nitric oxide (NO) production take place, thus making the phagolysosome very acidic and hydrolytic. Promastigotes, which are sensitive to acidic and hydrolytic environments, begin to be eliminated. However, as a survival strategy, the promastigotes begin to transform into the form of amastigotes, since the latter are more resistant (13, 27).

The infected macrophages stimulate the production of pro-inflammatory cytokines (IL-1, TNF, IL-18 and IL-12) and chemokines (CXCL10, CCL4, CCL8, CCL11 and CXCL8), which also act to kill the parasite. At the same time the adaptive immune response induce activation of T cells which produce a cellular immune response, and the B-lymphocytes will be involved in the production of antibodies (28, 29).

NATURAL KILLER CELLS

The NK cells are innate immune cells which correspond to 5-20% of circulating lymphocytes in the blood (30–32). These cells can be found in various locations in the body such as liver, bone marrow, and thymus (33) and can directly kill infected or modified tumor cells, in addition to their cytokine-producing function (30, 34).

NK cells are phenotypically known to express CD56 and CD16 and lack CD3 expression in humans. In mouse they express NK1.1 (NKR-P1C), NCR1 (NKp45/CD335) and CD49b, and there is no expression of CD56 by rodent NK cells (35–38). Natural Killer T cells (NKT) are a less frequent subpopulation (0.1-0.5% of peripheral blood leukocytes) of NKs characterized by CD3 and CD56 expression (38–41). NKT cells are T lymphocytes that possess characteristics of both these cells and NK cells, thus adding function of both cell types. There are two types of NKT cells: type I NKT or invariant NKT (iNKT), which express and invariant TCR with the Vα24-Jα18 segment associated with the Vβ11 chain, and type II NKT or non-invariant NKT cells, which express the TCR with varying TCRs on their cell surface (42–44). Among the characteristics of an NKT cell are the presence of the invariant TCR, expression of CD1d, and high production of cytokines, especially IFN-γ, TNF, IL-4, IL-10, and IL-13 (45–49).

WHY DOES THE STUDY OF NK AND NKT CELLS ARE IMPORTANT IN CUTANEOUS LEISHMANIASIS?

NK cells are part of the innate immune response and are known for their ability to kill infected cells, as well as to produce cytokines that act in the activation of other immune cells (**Figure 1**). For some time, studies have been trying to dissect

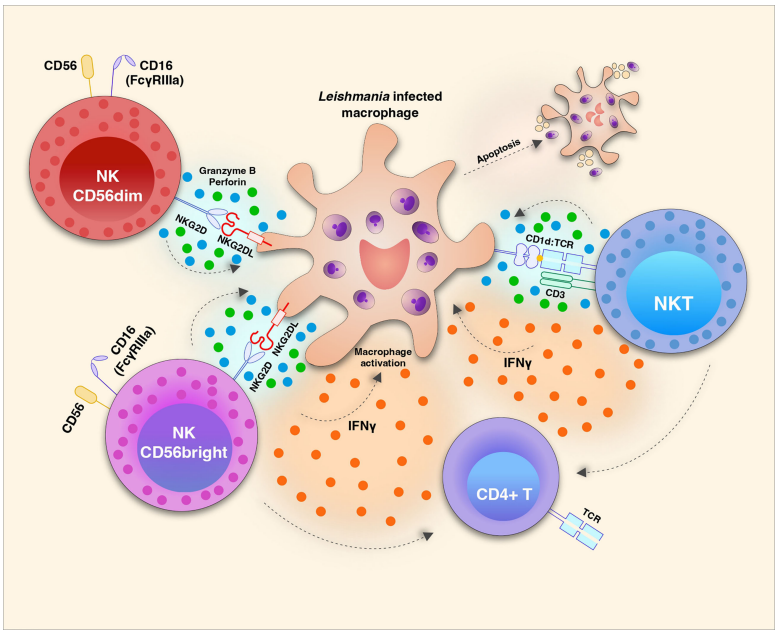

FIGURE 1 | Natural killer (NK) cell subsets play distinct roles during Leishmania infection. NKG2D/NKG2D ligand (L) interaction triggers the effector function of $CD56^{dim}$ (also $CD16^{+}$ in humans) NK cells to release cytotoxic granules with Granzyme b and Perforin which induces apoptosis of *Leishmania* infected macrophages. It is not yet clear tough if this mechanism restricts parasite growth or amplify amastigote proliferation. The same receptor-ligand interaction induces the activation of $CD56^{bright}$ NK cells which are strong producers of cytokines such as IFN-γ and TNF which in turn can act in the activation of macrophages and T cells. The potential recognition of glycolipid antigens present by CD1d to the invariant TCR of NKT cells may induce the production and release of IFN-γ and also apoptotic factors Granzyme B and Perforin.

the role of NK cells in CL, as well as their contributions to the cure or progression of the disease.

Some authors suggest that these cells act to fight infection due to their cytotoxic role, however, other authors suggest that these cells are also responsible for more collateral damage contributing to lesion exacerbation (50). To tackle this problem, a 5-year cohort study observed that even after clinical cure of CL, the patients' NK cells continue to produce Interferon-γ (IFN-γ), and this was associated with protection against CL (51). IFN-γ is key-cytokine for macrophage activation and consequent parasite clearance (52).

It has also been shown that the *in vitro* stimulation of peripheral blood mononuclear cells from healthy individuals with *Leishmania* antigen/peptides induces an increase in the frequency of NK cells specially (53, 54). On the other hand, Lieke and colleagues observed a decrease in NK cell proliferation in animal and human models using *L. major* and *L. aethiopica* where the promastigote form of *Leishmania* inhibited proliferation of isolated naive NK cells (55, 56).

NK cells are strong producers of cytokines and cytotoxic granules such as granzyme and perforin and surface markers such as NKG2D, triggering activation of other cells, increasing phagocytosis and parasite elimination (57). However, paradoxically, some works have shown evidence that these NK-derived effector molecules play a key role in the immunopathogenesis of CL, in addition to the inflammation developed. It was observed that patients with active lesion present a higher frequency of these cells, which in turn is directly associated with high levels of IFN-γ, Tumor Necrosis Factor (TNF), as well as granzyme A, granzyme B, granulysin and perforin. For this reason, it was found that most of the cytotoxic activity generated in CL is related to NK and not only to $CD8^+$ T cells (58–60). Another study showed that there is no change in the frequency of NK cells according to the period of infection, but in patients who are on treatment as well as in clinically cured patients there is a reduction in their degranulation potential (61).

There seems to be a divergent role of NK cells according to the clinical form that the patient displays. It has been seen that patients with the diffuse form of the disease have a lower frequency of NK cells, also exhibiting lower levels of cytokines such as IFN-γ and TLR expression such as TLR1, TLR2 and TLR6 (62, 63). This fact seems to be related to the severity of the disease. Unlike patients who present the localized form, who exhibit high levels of cytokines, besides a higher frequency of NK cells (63).

Experimental models have demonstrated that depleting NK cells in C57BL/6 mice through the administration of anti-asialo-GM1 or NK1.1 antibodies induces lesion exacerbation in the first weeks of infection. In addition, these animals showed swelling in the local tissue and higher parasite numbers when compared to normal animals (64). NK cell depletion has also been shown to lead to a reduction in IFN-γ levels, which may compromise Th1 response development (64, 65).

Studies on the role of NKT in *Leishmania* infection are still scarce (48). An *in vitro* study used monocyte-derived DCs generated from healthy donors' buffy coats observed that *Leishmania infantum* infected DCs increase the expression of CD1d, causing NKT cells to recognize these cells. In addition, the researchers observed that the percentage of NKT cells producing IFN-γ was twice as high as that of IL-4-producing cells. Thus, it can be seen that NKT cells can act both in the production of cytokines for the activation of other immune cells, such as T lymphocytes, as well as act in a cytotoxic way on infected cells that cannot be lysed by conventional NK cells (66).

Studies have observed subpopulations of NKT cells that express CD4 and CD8 markers, where these subpopulations have distinct functions. While CD4+ NKT cells are strong and potent producers of cytokines such as IL-2, IL-4 and IL-13, $CD8^+$ NKT cells act more aggressively in fighting infection, through their cytotoxic activity (67–69). Studies by Gumperz et al. (67) and Carvalho et al. (70) observed higher numbers of CD8+ NKT cells in healthy individuals (67, 70).

Recently it has been shown that antigen from *Leishmania braziliensis* is able to induce activation of NKT cells in peripheral blood mononuclear cells (PBMC) from patients with CL in addition to CD107a+ NKT cells, thus suggesting that these cells may be involved in inflammation and consequently lesion formation (59). This is confirmed by Ferraz and colleagues (2017) who observed the presence of these cells at the site of injury (71). CD107a is a surface marker found on NK cells as well as CD8+ T cells and is used to assess the degranulation of these cells, allowing one to investigate whether or not there is granule release and consequent lysis of infected cells (72, 73).

Currently, there is no consensus on the true function of NK and NKT cells in CL. The data presented by the studies suggest that NK cells appear to contribute strongly to a toxic environment and consequently to the development of lesions. At the same time, these cells seem to be crucial for the development of a cellular immune response, in view of their high production of IFN-γ, which helps in the activation of cells of the adaptive immune response. Studies to understand the factors that lead NK cells to generate a balance at the site of injury are strongly recommended.

Thus, more comprehensive research on these cells and their interaction with *Leishmania* is urgent. For example, single-cell RNA sequencing studies are necessary and can help understand the contribution of NK cells to lesion formation or resolution in CL. Moreover, those studies would foster the development of new treatments, diagnostics and vaccines to effectively combat CL.

AUTHOR CONTRIBUTIONS

MC, RS and MB-D-C wrote the manuscript. VP contributed to the discussion of the draft and made final corrections. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by UFPE-Propesqi nº 12/2021. MC is the recipient of a Ph.D. fellowship from FACEPE.

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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 19 August 2022
DOI 10.3389/fimmu.2022.914598

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 07 April 2022
ACCEPTED 21 July 2022
PUBLISHED 19 August 2022

CITATION
Pohl K and Cockburn IA (2022) Innate immunity to malaria: The good, the bad and the unknown.
Front. Immunol. 13:914598.
doi: 10.3389/fimmu.2022.914598

Innate immunity to malaria: The good, the bad and the unknown

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Malaria is the cause of 600.000 deaths annually. However, these deaths represent only a tiny fraction of total malaria cases. Repeated natural infections with the causative agent, *Plasmodium* sp. parasites, induce protection from severe disease but not sterile immunity. Thus, immunity to *Plasmodium* is incomplete. Conversely, immunization with attenuated sporozoite stage parasites can induce sterile immunity albeit after multiple vaccinations. These different outcomes are likely to be influenced strongly by the innate immune response to different stages of the parasite lifecycle. Even small numbers of sporozoites can induce a robust proinflammatory type I interferon response, which is believed to be driven by the sensing of parasite RNA. Moreover, induction of innate like gamma-delta cells contributes to the development of adaptive immune responses. Conversely, while blood stage parasites can induce a strong proinflammatory response, regulatory mechanisms are also triggered. In agreement with this, intact parasites are relatively weakly sensed by innate immune cells, but isolated parasite molecules, notably DNA and RNA can induce strong responses. Thus, the innate response to *Plasmodium* parasite likely represents a trade-off between strong pro-inflammatory responses that may potentiate immunity and regulatory processes that protect the host from cytokine storms that can induce life threatening illness.

KEYWORDS

malaria, sporozoites, innate immunity, blood stages, pattern recognition receptors, pathogen-associated molecular patterns, *Plasmodium*

Introduction

Malaria remains a major cause of morbidity and mortality worldwide with an estimated 627,000 deaths in 2020 (1), though this represents a small fraction of the over 200 million clinical cases per year. Moreover, in high transmission areas, 40-70% of individuals can harbor malaria parasites in their blood as asymptomatic infections (2). Thus, while the overall burden of disease is high, case fatality is low. Moreover, even though asymptomatic

individuals have lower levels of infection than individuals with severe or mild infection, immunity does not control infection and parasite biomass is high. However, in semi-immune individuals this parasitaemia does not induce the florid and life-threatening innate response associated with bacteraemia and sepsis. Thus, a key outstanding question is how malaria infections can be tolerated by the immune system, while also being controlled. A further outstanding question is that while blood stages appear to be largely tolerated by the immune system, other lifecycle stages - notably the sporozoite and liver stages are rapidly sensed by the innate response and can induce robust immune responses.

Human malaria is caused by one of five species of the single-celled eukaryotic genus *Plasmodium;* with the highest disease burden caused by *P. falciparum (*3, 4). *Plasmodium* parasites have a complex lifestyle that entails sexual reproduction in the *Anopheles* vector and asexual reproduction in the human host [reviewed elsewhere (4, 5)]. Human infection begins when sporozoites are delivered into the skin by the bites of an infected *Anopheles* mosquito. Sporozoites travel from the skin *via* the bloodstream to the liver, where they invade hepatocytes. During the liver stages, a single parasite gives rise to tens of thousands of merozoites which are then released into the bloodstream initiating the symptomatic blood stage of a *Plasmodium* infection by replicating inside of erythrocytes. Importantly, while only tens to hundreds of sporozoites are injected into the dermis per mosquito bite, one successfully developing sporozoite is enough to cause blood stage infection with an average of 7 x 10^{11} blood-borne parasites (6). A subset of these blood stages will develop into sexual stage gametocytes which can continue the cycle of infection if taken up during blood feeding by another mosquito (5).

Symptoms of malaria range from none in partially immune individuals, to cyclic fever and to severe manifestations leading to death. However only around 1% of clinical infections lead to severe malaria, most often in immunological naïve individuals (3). Accordingly, in endemic areas children under 5 years of age are most affected and most likely to suffer from severe malaria and death (3). Semi-immunity, characterized by the host's ability to tolerate and - to an extent - suppress blood stage infection, is acquired over the course of several disease episodes (7, 8). This semi-immunity is most often attributed to antibody-mediated control of parasitemia (resistance) (7) and to an ability to tone down overarching inflammatory responses during peak infection (tolerance) (8). Importantly, even numerous blood stage infections do not induce sterile immunity and people living in endemic areas are repeatedly infected throughout their lives. This fact has led to the notion that blood stage infection induces a form of unnatural and ineffective immunity.

While blood stage infection induces strong inflammatory responses in naïve individuals but not sterile immunity, it has long been known that attenuated sporozoites can induce sterile immunity, which has been a major model for vaccination. This is true whether sporozoites are attenuated by irradiation, drug control of subsequent blood stage infection, or knockout of key genes required for progression from liver stages to blood stages (9–11). However, sporozoite vaccines are hampered by the logistical challenges associated with the preparation of large amounts of sporozoites, the possibility of breakthrough infections and reduced efficacy in endemic settings (12, 13). Nonetheless, compared to blood stage infection - relatively small numbers of sporozoites induce a strong adaptive immunity characterized by high titers of affinity matured antibodies and strong CD8 T-cell responses (14). In this regard, an outstanding question in the field has been how different life cycle stages of the same parasites induce such different adaptive immune responses.

Innate immunity to pre-erythrocytic stages

While sporozoites are evolutionarily optimized for their journey to the liver, only a few manage to infect hepatocytes with the remaining sporozoites being cleared by cells of the innate immune system. These unsuccessful sporozoites likely contribute to innate immune activation and are a source of antigen for T and B cells (15). *In vivo* tracking of fluorescent sporozoites after intradermal injection revealed that similar numbers of viable *P. berghei* sporozoites reached the liver or remained in the skin (16). Importantly, sporozoites were found in subcapsular zones of skin draining lymph nodes (dLNs) before being taken up into dLN resident $CD8^+$ Dendritic cells (DCs) which efficiently prime $CD8^+$ T-cells (16, 15),. The important role for mouse $CD8^+$ cDC1s in priming sporozoite-specific $CD8^+$ T-cells was also shown in $Batf3^{-/-}$ mice which lack cDC1s (15, 17, 18). In addition, targeted expression profiling of dLNs 24 h after sporozoite injection revealed elevated expression of CXCL9, CXCL10, Granzyme B and IFNγ (16). Work done with the *P. yoelii* model further suggested that priming in dLNs after subcutaneous sporozoite injection is sufficient to induce protective immunity (19, 20). IFNγ, dendritic cells as well as $CD4^+$ and $CD8^+$ T-cells also play important roles during the induction of immunity, while $CD8^+$ T-cells were most important for protection from challenge later on (20).

Beyond animal models, controlled human malaria infections, often as part of clinical trials of whole sporozoite vaccines have also provided insights into protective immune responses. Interestingly, one of the strongest correlates of protection in sporozoite vaccinated individuals was increased frequencies of $V\gamma9^+$ $V\delta2^+$ T-cells at baseline and 2 weeks after final immunization (14). In line with these findings, RNA sequencing of PBMCs isolated from vaccinated individuals identified that elevated expression of two genes encoding γδTCRs (TRDV2 and TRGV9) at 3 days after the last vaccination were associated with protection from mosquito-bite challenge (18). Gamma-delta T cells are an innate-like

population of T-cells that express a limited repertoire of γδTCRs. Only very few ligands for γδTCRs have been identified so far (21), however, even without vaccination, γδT-cells in *in vitro* *Pf*SPZ-stimulated PBMCs produced IFNγ, indicating the presence of a γδTCR ligand in sporozoites (14). In mouse models, depletion of γδT-cells during sporozoite vaccination ablated protective immunity mediated by CD8+ T cells further supporting a role for this population in the induction of adaptive immune responses (18).

Once in the liver, intrahepatic stages of the rodent *P. berghei* parasite induce the expression of type I IFNs (22). This response appeared dependent on parasite replication, as irradiated parasites induced markedly reduced levels of IFN expression (22). This response was abrogated in mice deficient for Mitochondrial antiviral-signaling protein (MAVS) (22). MAVS is the adaptor protein for the cytosolic RNA sensors Melanoma differentiation-associated protein 5 (MDA5) and Ritionic acid inducible gene I (RIG-I) (23). Upon activation, for example during viral infection, MAVS induces the expression of Type I IFN and thus contributes to cell intrinsic and extrinsic defense against pathogens (23). Interestingly, cytokine production in response to intrahepatic parasites was only partially reduced in MDA5 deficient mice and not affected in Rig-I deficient animals, suggesting the existence of another cytosolic pattern recognition receptor (PRR) capable of detecting parasite pathogen associated molecular patterns (PAMPs). The cell type that senses *Plasmodium* RNA was not identified and could be either innate immune cells or the hepatocyte itself (Figure 1) (22). Simultaneously, a second group suggested that type I IFN may recruit IFN-gamma producing NK cells to the liver to reduce hepatic parasite burden (24). A possible mechanism through which IFNγ can mediate a reduction of liver stage burden could be an autophagy pathway that kills intrahepatic parasites (25, 26)

In vivo experiments can be complemented by *in vitro* co-culture experiments of sporozoites with either primary or cultured innate immune cells. However, such studies are hampered by difficulties obtaining pure populations of sporozoites. Initial experiments co-culturing *P. yoelii* sporozoites with mouse peritoneal macrophages revealed that sporozoites induced a respiratory burst in macrophages (27). Interestingly, frequencies of activated macrophages were lower when using salivary gland sporozoites than when immature oocyst-like sporozoites were used (27). Shortly after, Vanderberg and colleagues (28) characterized sporozoite macrophage interactions *in vitro* using live cell imaging and discovered multiple modes of host-parasite interaction. Importantly, sporozoites were shown to actively penetrate and subsequently egress from macrophages, a process that in some cases led to the destruction of the innate cells. This traversal process has subsequently been shown to be of great importance for sporozoites *in vivo* to leave the dermis after deposition through a mosquito bite and traverse endothelial cells and

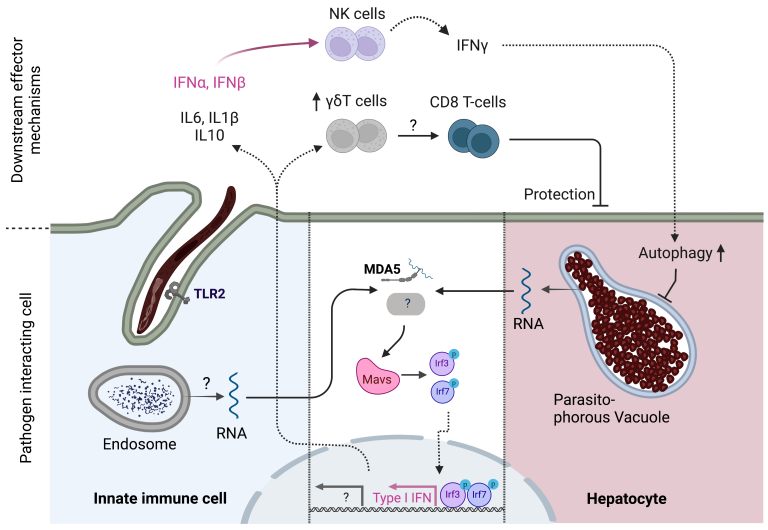

FIGURE 1
Innate immune response to pre-erythrocytic stages of *Plasmodium* parasites. Cytoplasmic *Plasmodium* RNA is sensed by MDA5 which signals *via* MAVS. MAVS activation ultimately leads to the phosphorylation of transcription factors IRF3 and IRF7 which drive the expression of Type I IFN genes such as IFNα and IFNβ. IFNα,β recruit NK cells to the liver which produce IFNγ which in turn increases autophagy pathways in hepatocytes. Another, yet unidentified, PRR might signal *via* MAVS to enhance this response. The cell type of origin of the initial Type I IFN response has not been identified and could either be infected hepatocytes or tissue resident innate cells that have taken up parasite material. TLR2 has been shown to be capable of sensing sporozoites, leading to reduced liver stage burden and enhanced inflammatory gene expression in mice. In addition, γδT-cells have been linked to favorable vaccination outcomes in humans and play a role in inducing a protective CD8 T-cell response in concert with $CD8^+$ cDC1s in mice. Created with Biorender.

Kupffer cells prior to infecting hepatocytes (29–31). Host-cell traversal also induces the loss of macrophage plasma membrane integrity (32) and activates Kupffer cells (33).

In addition to cytosolic RNA sensors identified *in vivo*, Toll-like receptor 2 (TLR2) has also been identified as an innate sensor of sporozoite infection from *in vitro* studies (34). TLR2 belongs to the Toll like receptor family whose members can sense a range of PAMPs in the extracellular space or the endosome (35). Most TLRs signal *via* MyD88 to induce strong NF-κB driven pro-inflammatory gene expression and the secretion of cytokines such as IL6 and TNFα (35). It was further shown that TLR2 deficient mice had higher liver stage burden after intravenous sporozoite injection and failed to express inflammatory cytokines in the liver. However it is not clear whether TLR2 acts in concert with MDA5 and MAVS to induce a hepatic type I IFN response (34). Another recent *in vitro* study characterized human innate cell co-culture with *P. berghei* and *P. falciparum* sporozoites, showing that the parasites were readily taken up into monocyte derived macrophages and DCs, which in turn produced IL-6, IL-1β and IL10 (36). Overall, sporozoites appear to be potent inducers of pro-inflammatory immune responses that potentiate adaptive immunity, however, both the interacting cells and the ligand-receptor pairs that mediate these responses remain poorly characterized.

Innate immunity to blood stage infection

While sporozoites appear to be potent stimulators of both innate and adaptive immune responses especially when considered on a per parasite basis, the pre-erythrocytic stages of malaria infection are clinically silent because the number of invading parasites is so small. In contrast, blood stage infection is responsible for the disease manifestations of malaria which can be characterized by sepsis-like excessive inflammation in naïve individuals (3, 37). However, many immunopathology mechanisms during severe blood stage infection target specific tissues and rely on tissue-specific host-parasite interactions (38). Key virulence factors for malaria include various families of antigenically variant surface antigens (VSAs), including the var (encoding PfEMP1), rif (encoding RIFIN) and stevor (encoding STEVOR) gene families of *P. falciparum* that encode around 60, 200 and 30 highly polymorphic genes, respectively (39–41). In particular, the PfEMP1 family of surface receptors allow infected erythrocytes to adhere to endothelial cells allowing for the sequestration to prevent their removal *via* the spleen. These adhesion processes are associated with severe disease (42), in particular cerebral malaria due to obstruction of blood vessels in the brain. In addition to var genes, RIFINs also play roles in immune modulation. Three human receptors have been found to bind to *P. falciparum* RIFINs; LAIR1 (43, 44), LILRB1 (45, 46) and LILRB2 (47). All of these receptors belong to the Ig superfamily, contain intracellular immunoreceptor tyrosine-based inhibitory (ITIM) motifs and are broadly expressed by myeloid cells and lymphocytes (48–50). ITIM motifs downregulate cell activation by antagonizing activation signals in many different immunological contexts, such as NK-cell activation, T and B cell activation and myeloid cell activation after PRR engagement (51).

Seminal work showed that PfEMP1 can also be used by infected erythrocytes to bind to human DCs *via* CD36 resulting in the inhibition of LPS-induced maturation and a reduced ability of DCs to induce T-cell proliferation (52). In human monocytes, *Pf*EMP1 expressing parasites also induced weaker inflammatory cytokine expression, corroborating the immune modulating role of *Pf*EMP1 (53). Interestingly, More recent work characterizing human dendritic cell responses to blood stage parasites also found atypical activation patterns with a marked absence of inflammatory cytokine production and low co-stimulatory molecule expression (54). Nonetheless, DCs stimulated with intact parasitized erythrocytes were able to potently activate $CD4^+$ T-cells *in vitro* marked by the induction of high levels of IFNγ and TNFα (54). A similar activation phenotype was also observed in DCs isolated from individuals from endemic countries (54, 55).

Rodent malaria models have also provided key insights into innate immune interactions with blood stage parasites, circumventing some of the limitations of *in vitro* studies that use long-term cultured *P. falciparum* strains. In agreement with human data, mouse bone marrow derived DCs also show reduced maturation capacity upon stimulation with LPS, when they were pre-treated with *P. yoelii* blood stage parasites *ex vivo* (56). In addition, it was shown that DCs stimulated with *ex vivo* purified *P. yoelii* infected erythrocytes produced soluble mediators that reduced $CD8^+$ T-cell activation, giving rise to the idea that blood stage infection could suppress immune responses to pre-erythrocytic stages. Later work, however, found no defect in infected erythrocyte-induced DC maturation (57), and confirmed the ability of DCs to phagocytose parasites and induce long lasting immune responses (58). In agreement with this, *in vivo* studies in the *P. berghei* ANKA model, which is characterized by T-cell dependent cerebral cytotoxicity (59), have shown that depletion of conventional DCs ameliorated T-cell mediated immune pathology, while parasite control remained unaffected (60, 61). These contrasting findings highlight that DC biology during blood stage malaria is still incompletely understood and important differences are present between rodent and human host-pathogen pairs likely determined by differences in the VSAs on infected erythrocytes.

In addition to understanding cellular host-parasite interactions, research into innate immunity to blood stages has focused on identifying parasite immune stimulatory ligands and

their host cell receptors (Figure 2). The first candidate parasite PAMPs identified were Glycosylphosphatidylinositols (GPIs), which are complex lipid, carbohydrate and phosphate containing molecules that are common in all eukaryotic life and function to anchor proteins to membranes (62). *Plasmodium* GPIs contain conserved molecular features that are distinct from human GPIs (62). Early studies identified *P. falciparum* GPIs as immune stimulatory ligands for innate cells that induced the production of TNFα and IL-1β in macrophages and resulted in immune pathology in mice upon injection (63). TLR2 was later identified to be the receptor for GPIs of several protozoan parasites including plasmodia through the formation of heterodimers with either TLR1 or TLR6 (64, 65).

A second well-researched blood stage PAMP is hemozoin. Through digestion of haemoglobin, intraerythrocytic *Plasmodium* parasites generate toxic heme, which can produce free radicals in the parasite digestive vacuole. To detoxify heme, *Plasmodium* parasites convert it into an insoluble crystallized form, called hemozoin which can be released into the bloodstream during rupture of the infected red blood cell (66). Hemozoin has been studied extensively in its ability to induce innate activation and has led to many confusing findings mainly due to its ability to complex many biological entities including lipids and nucleic acids that themselves can activate PRRs (67, 68). The study of synthetic hemozoin that is free of contaminants in *in vitro* systems has ultimately led to the idea that hemozoin itself is relatively inert to innate cells, but functions by delivering PRR ligands to their respective receptors (69). However, there are also reports that challenge this notion and attribute the release of numerous inflammatory cytokines such as IL6 and IL1ß to the sensing of hemozoin through the NLRP3 inflammasome in mice (70–72). Inflammasomes are multi-protein complexes that assemble in response to environmental triggers in the host cell cytosol. Once assembled, these complexes enzymatically cleave pro-forms of the highly inflammatory cytokines IL-1ß and IL18 into their respective bioactive forms (73). Inflammasomes thus play critical roles in pathogen defense but have also been implicated in autoimmune disorders (73).

The investigation of hemozoin as a malaria PAMP is tightly connected to the study of plasmodial DNA as activating ligand of innate immune cells. As such, it was first thought that hemozoin is the activating ligand of TLR9 (74, 75). However, subsequent studies in which hemozoin was prepared free of DNA contamination and used to stimulate mouse bone marrow derived DCs showed that in fact *Plasmodium* DNA was the TLR9 ligand and that it was bound to hemozoin (69). The ability of human TLR9 to sense plasmodial DNA has been confirmed

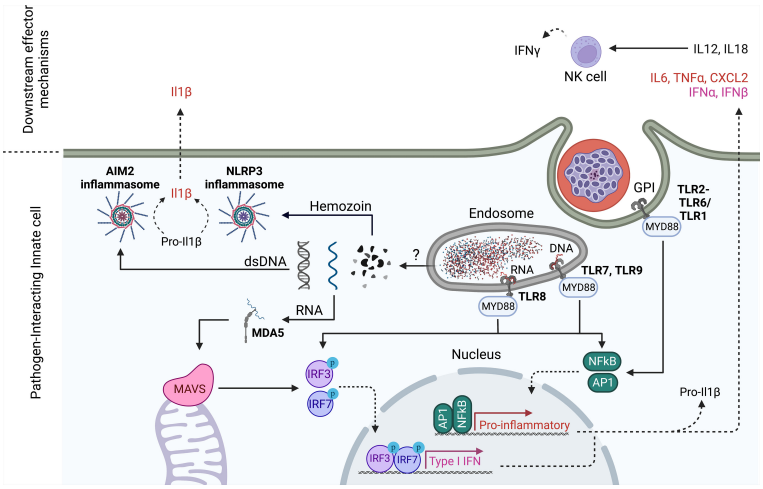

FIGURE 2
Innate sensing mechanisms of *Plasmodium* blood stages. TLR2 senses *Plasmodium* GPIs on the cell surface, while TLR7 and 9 recognize *Plasmodium* DNA in the endosome. In humans, TLR8 also senses degradation products of *Plasmodium* RNA. TLR engagement drives pro-inflammatory gene expression and a Type I IFN response (only TLR7, 8,9) that is dependent on MyD88 signaling. *Plasmodium* nucleic acids escape from the lysosome probably through direct association with hemozoin. In analogy to pre-erythrocytic stages, *Plasmodium* RNA in the cytoplasm is detected by MDA5 leading to MAVS activation and downstream Type I IFN gene expression. In addition, cytoplasmic double stranded (ds)DNA is sensed by AIM2 while Hemozoin is sensed by NLRP3, each leading to inflammasome assembly and enzymatic cleavage of pro-inflammatory mediators like Pro-IL1β. Akin to pre-erythorcytic stages, NK cells have been shown to produce IFNγ downstream of PRR recognition of parasite PAMPs. In *ex vivo* culture systems of human cells, NK cells have been shown to produce IFNγ in response to innate cell produced IL18 and IL12 which were dependent on TLR8. Created with Biorender.

by independent groups (76, 77). Interestingly, hemozoin seems to have an adjuvating effect when it is complexed with DNA (69), supposedly by allowing DNA access to the cytosol where it also activates cytosolic DNA sensors such as AIM2 (78). However, it is still unclear how hemozoin escapes from endosomes. Upon ligand engagement, endosomal DNA sensors TLR7 and TLR9 predominantly initiate a type I IFN response through the activation of IRF transcription factors. In addition, type I IFN production was also shown to be induced after cGAS-mediated detection of *Plasmodium* DNA in the cytoplasm (79). Again, the authors show that DNA access to the cytosol is mediated by complex formation with hemozoin (79).

RNA sensing mechanisms and their role during disease have also been studied. While in mice, MDA5 seems to be activated during blood stage *P. yoelii* infection to signal *via* MAVS the production of type I IFNs (80), the activating ligand for MDA5 has not clearly been identified yet. Interestingly, the authors found that ablating IFN production after cytosolic DNA or RNA sensing protected mice from lethal *P. yoelii* challenge, while abrogating endosomal nucleic acid sensing through TLR7 and TLR9 did not (80). More recently, Coch et al (81) discovered that human TLR8 senses *Plasmodium* RNA in the endosome leading to robust induction of IL-1β in the human THP-1 cell line that was abrogated in TLR8 deficient THP-1 cells. Shortly after, RNAse T2 was shown to degrade endosomal *Pf*RNA prior to sensing of degradation products by TLR8 (82). When human PBMCs were stimulated with blood stage parasites, TLR8 contributed to mounting a partly NK-cell dependent IFNγ response (81), which is in line with earlier findings that the TLR8 adaptor MyD88 is necessary for *P. falciparum* blood stage induced IFNγ production by NK cells (83).

Concluding remarks

Blood stage malaria manifests as a severe inflammatory disease in naïve individuals. In animal models, genetic ablation of innate sensing pathways often offer survival benefits pointing towards a role for dysregulated inflammatory signaling in innate cells in malaria pathology (80). However, reductionist co-culture systems with intact parasites generally seem to reveal nuanced responses of innate cells that often show a lack of, or reduced inflammatory signaling (54), unless parasites are added in high quantities or stimulatory ligands are purified.

To understand this seeming contradiction, a biomass comparison may be made between a *Plasmodium* blood stage infection, sporozoite immunization and sepsis caused by *E. coli* (Table 1). A blood stage *Plasmodium* infection can involve hundreds of billions of parasites with a total biomass of several grams. Nonetheless, such infections are frequently tolerated by the host, even in naive or semi-immune individuals. In contrast, a septicemic *E. coli* infection can induce a life-threatening cytokine storm with one millionth of the amount of antigen. This comparison argues that on a per-pathogen and per gram biomass basis, blood stage parasites have a relatively low inflammatory capacity as compared to bacteria. Interestingly, comparatively low numbers of sporozoites can induce sterile immunity when used as a vaccine, while 6 orders of magnitude more blood stage parasites do not suffice.

Understanding the mechanistic basis for this difference could be highly valuable. An explanation for these observations can likely be found considering the pressure that evolution exerts on host parasite interactions. This pressure likely favored different outcomes for pre-erythrocytic and blood stage parasites, especially considering their respective roles during the parasite life cycle: While only a single sporozoite needs to productively infect a hepatocyte to complete its mission, blood stage parasites need to keep proliferating in the blood for long periods of time to ensure successful uptake of gametocytes into a feeding mosquito.

Thus, blood stage parasites were subject to high evolutionary pressure to survive host sensing pathways to avoid destruction by innate or adaptive immunity. On the other hand, sporozoites might have evolved to very efficiently reach host hepatocytes while being much less manipulative regarding innate sensing pathways. Their high success rate allows only very few sporozoites to be deposited into the skin during a mosquito blood feed, limiting the amount of PAMPs to be detected and the amount of antigen available for the induction of protective immunity.

Ultimately, a deeper understanding of innate pathways that are activated by the parasite's life cycle stages and their respective downstream contributions towards beneficial *vs.* detrimental inflammatory and adaptive immune responses will be needed to guide both treatment and prevention strategies of the future.

TABLE 1 Comparison of pathogen numbers and biomass during *P. falciparum* blood stage infection, sporozoite vaccination and *E. coli* mediated sepsis.

	Blood stage infection	Sporozoite vaccine	E. coli sepsis
Total number of pathogens in blood	7×10^{11} [ref (6)]	2×10^{5} [ref (84)]	4×10^{6} [ref (85)]
Weight per pathogen	34.6 pg [ref (86)]	34.6 pg [ref (86)]	5 pg [ref (87)]
Total pathogen biomass	24.200.000 µg	6.92 µg	19.5 µg

Pathogen numbers were curated and calculated from references indicated in the table for an average human female. Weight for average trophozoites was approximated using volumetric measurements from indicated reference (49 fl) and the dry weight to water ratio of bacteria (0.22). In rough approximation, the same weight was assumed for sporozoites.

Author contributions

KP drafted the original manuscript, prepared the figures and redrafted the manuscript. IC reviewed, redrafted and edited the manuscript for submission. All authors contributed to the article and approved the submitted version.

Funding

KP was supported by PhD studentship from the Deutsche Forschungsmeinschaft Crossing Boundaries Molecular Interactions in Malaria International Research Training Group Program (IRTG2290).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 31 August 2022
DOI 10.3389/fimmu.2022.941977

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 12 May 2022
ACCEPTED 01 August 2022
PUBLISHED 31 August 2022

CITATION
Wu Y, Duffey M, Alex SE, Suarez-Reyes C, Clark EH and Weatherhead JE (2022) The role of helminths in the development of non-communicable diseases. *Front. Immunol.* 13:941977. doi: 10.3389/fimmu.2022.941977

The role of helminths in the development of non-communicable diseases

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Non-communicable diseases (NCDs) like cardiovascular disease, chronic respiratory diseases, cancers, diabetes, and neuropsychiatric diseases cause significant global morbidity and mortality which disproportionately affect those living in low resource regions including low- and middle-income countries (LMICs). In order to reduce NCD morbidity and mortality in LMIC it is imperative to understand risk factors associated with the development of NCDs. Certain infections are known risk factors for many NCDs. Several parasitic helminth infections, which occur most commonly in LMICs, have been identified as potential drivers of NCDs in parasite-endemic regions. Though understudied, the impact of helminth infections on the development of NCDs is likely related to helminth-specific factors, including species, developmental stage and disease burden. Mechanical and chemical damage induced by the helminth in combination with pathologic host immune responses contribute to the long-term inflammation that increases risk for NCD development. Robust studies from animal models and human clinical trials are needed to understand the immunologic mechanisms of helminth-induced NCDs. Understanding the complex connection between helminths and NCDs will aid in targeted public health programs to reduce helminth-induced NCDs and reduce the high rates of morbidity that affects millions of people living in parasite-endemic, LMICs globally.

KEYWORDS

helminths, non-communicable diseases, cestodes, trematodes, nematodes, immunity

The global impact of non-communicable diseases

Non-communicable diseases (NCDs) such as cardiovascular disease, chronic respiratory diseases, cancers, diabetes, and neuropsychiatric diseases are now the most common causes of morbidity and mortality globally, including in low- and middle-income countries (LMICs) (1). NCDs are responsible for over 71% of all deaths

worldwide and 1.6 billion disability adjusted life years (DALYs) (2). The rise in NCDs is likely multifactorial due to changes in lifestyle including reduced physical activity and non-nutritious diets, increased ability to diagnose NCDs in resource limited regions around the world, lengthening of the human life expectancy and enhanced control efforts of communicable diseases (3, 4). Though NCDs are common in all countries regardless of income level, LMICs have higher NCD-related mortality rates compared to those living in high-income countries (HICs); in fact, 80% of NCD-related deaths occur in LMICs (5). Furthermore, while NCD-related mortality in HICs is associated with older age groups, NCD-related deaths in LMICs are concentrated in younger adults (the 30-69 years old age group). Strikingly, 85% of NCD-related deaths in persons 30-69 years old occur in LMIC (6). These differences between LMICs and HICs demonstrate the profound impact of social determinants of health, such as limited resources, poor health infrastructure and poverty, on global health outcomes related to NCDs and the need to identify region-specific modifiable risk factors for NCDs (7).

Generally, factors such as high blood pressure, smoke exposure, poor glucose control, and obesity are associated with increased risk of NCDs. However, major differences exist between NCD risk factors in HICs compared to LMICs. While diet composition is a standard risk factor for the development of NCDs in both HICs and LMICs, cigarette smoking is a much larger driver of NCDs in HICs compared to LMICs. In contrast, environmental drivers of NCDs (e.g., use of indoor biomass fuel and infectious diseases) are more common in LMICs than HICs. Furthermore, because infectious diseases remain more prevalent in LMICs, NCDs attributed to infections are associated with higher DALYs in LMICs compared to HICs (8).

Several infectious pathogens are recognized as substantial contributors to the development of various NCDs. Most of these pathogens are more common in LMICs than in HICs. For instance, Hepatitis B virus and Hepatitis C virus are well-known risk factors for liver cirrhosis and cancer. Human Papilloma virus (HPV) and the bacteria *Helicobacter pylori* can also cause cancer (9–11). Many infectious respiratory pathogens are known to result in chronic respiratory disorders including chronic obstructive respiratory disease (COPD; a potential late consequence of tuberculosis, histoplasmosis, etc.) and asthma (which can manifest after childhood Respiratory Syncytial Virus infection) (12, 13). Though less well-known, parasitic infections—from protozoa like *Trypanosoma cruzi* (an infection that manifests as Chagas disease, leading to the development of cardiomyopathy or GI disease in 30% of infected individuals) and *Trypanosoma brucei* (an infection that causes sleeping sickness, leading to chronic neurologic disease in Sub-Saharan Africa) to helminths—can also increase one's risk for NCDs (14). This review will focus on helminth infections that drive the development of NCDs in endemic LMICs (Figure 1). Improving treatment and control of these helminth infections will be an important part of reducing global NCD morbidity and mortality.

Helminths as a cause of NCDs

Helminths are multicellular parasitic worms that disproportionately infect persons living in poverty-stricken regions of the world. While by far more prevalent in tropical LMICs, helminths prosper in nearly all impoverished regions with climates supportive of the parasitic life cycle (15), including in the southern United States (U.S.). Helminth infections generally do not cause high mortality rates but contribute to high morbidity and subsequent DALYs. Heavy helminth infections can lead to childhood malnutrition, growth restriction and neurocognitive impairment that impact school attainment and work productivity into adulthood (16). Additionally, helminth infections cause clinical manifestations and sequelae that are unique consequences of the individual helminth's life cycle. For this reason, several helminth infections can lead to NCDs including cardiovascular disease, lung disease, cancer and neuropsychiatric disease (Figure 1). On a community level, helminth-induced NCDs could have detrimental consequences on the economic growth in helminth endemic regions, keeping the entire community in poverty.

Nematodes

Soil-transmitted helminths (*Ascaris*, hookworm, *Trichuris*)

Ascariasis ("roundworm"), caused by either *Ascaris lumbricoides* or *Ascaris suum*, is the most common human helminth infection (17). The life cycle of *Ascaris* spp. begins with ingestion of *Ascaris* eggs from the contaminated environment. The larvae hatch in the digestive tract and migrate to the liver followed by the lungs *via* the systemic circulation. In the lungs, the larvae infiltrate the pulmonary parenchyma through the endovasculature and mature into late stage larvae prior to ascending the bronchotracheal tree and returning to the intestines to develop into adult worms (18). The larval migratory phase through the lungs can also cause prolonged mechanical and chemical lung damage leading to functional changes including asthma and chronic obstructive pulmonary disease (COPD). Animal models have shown that pulmonary larval migration causes a coordinated recruitment of eosinophils and neutrophils as well as type 2 T helper cells (Th2) that secrete type-2 cytokines (IL-4, IL-5, IL-13) and Th17 cells. (18, 19). These type-2 and type-17 immune responses inhibit larval development and reduce parasite burden, but lead to extreme allergic airway disease, an asthma phenotype (19, 20).

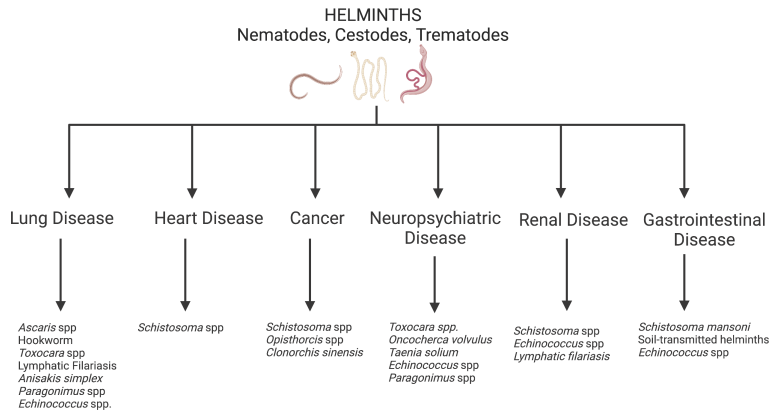

FIGURE 1
Helminth induced non-communicable diseases. Created with BioRender.com.

Data from these mouse models have been corroborated in human clinical studies. Previous studies have not only shown an increased risk of asthma (21) but increased asthma severity and need for hospitalization in children exposed to *Ascaris* in endemic regions (22). Together, these data suggest ascariasis is likely a major environmental cause of asthma in endemic regions. Moreover, mice previously infected with *Ascaris* have increased lung volumes, lung compliance and alveolar mean linear intercept (MLI) up to 9 months post infection, representing an emphysematous COPD phenotype. Mechanistic analysis reveals enhanced secretion of matrix metalloproteinase (MMP)-12 from alveolar macrophages, a key mediator in COPD pathology. Though no human clinical studies have yet been done to evaluate the association of COPD with *Ascaris* larval migration, *Ascaris* infection in pigs–a natural *Ascaris* host with similar lung-body weight ratio as humans–can lead to chronic paroxysmal coughing and expiratory dyspnea, suggesting *Ascaris* larval migration can cause chronic lung disease.

Along with the chronic lung disease induced in *Ascaris* mouse models, chronic vascular damage has also been described (23). Studies of mouse lungs 9 months post infection demonstrate pulmonary vascular permeability, erythrocyte extravasation and hemosiderin-laden macrophages, as well as chronic anemia. Anemia is a common clinical manifestation in children with ascariasis classically thought to be secondary to malabsorption of nutrients during the adult intestinal stage (24). However, as mice do not develop patent adult worm infection, the chronic bleeding in the lungs was likely a source of the persistent anemia. After migration through the lungs, *Ascaris* larvae mature and develop into adult worms in the intestines where they live up to 1-2 years. Clinical manifestations of intestinal ascariasis are usually subtle. However, hepatobiliary disease can develop from adult worms invading the biliary tract which can result in biliary strictures, biliary cirrhosis and atrophy of the liver progressing to end stage liver disease (25).

Hookworms, *Ancylostoma duodenale* and *Necator americanus*, infect humans through skin penetration of L3 filariform larvae (infectious stage) found in the soil. L3 larvae travel in the circulatory system to the lungs and enter the alveoli, transcend the trachea and are swallowed back to the gastrointestinal tract, where they molt into L4 larvae and develop into adult worms (26, 27). While there are no clinical studies suggesting hookworm infection is a risk factor for asthma (28, 29), a mouse model of hookworm (using *Nippostrongylus brasiliensis*) found larval migration through the host lungs and mucosal damage increased expression of Trefil factor 2 (TFF2), a central effector molecule in asthma. TFF2 orchestrated chronic lung repair but drove IL-13 associated allergic inflammation and airway hyperreactivity while promoting lung fibrosis after *N. brasiliensis* larval migration (30). In a chronic mouse model of *N. brasiliensis*, larval migration through the lungs induced an emphysematous COPD phenotype (31) as well as persistent hemosiderin-laden macrophages further suggesting the chronic impact of acute hookworm larval migration through the host lungs. The most serious known consequence of hookworm infection is chronic iron-deficiency anemia which can have devastating outcomes in pregnant persons (i.e., pregnancy loss and premature labor) and young children (i.e., cognitive delays) (32, 33). Overwhelming evidence supports that iron deficiency anemia is a direct result of intestinal hookworms infection. Adult hookworms attach to the intestinal mucosa using buccal plates and secret anticoagulant peptides that aid in blood extravasation and digestion utilized for nutrient acquisition by the worm (34). Heavy adult hookworm burden can lead to losses of over 1 mL of blood per day depending on the species of hookworm and the worm burden (35).

Trichuriasis is caused by oral ingestion of *Trichuris trichiura* ("whipworm") eggs from the contaminated environment (36). After ingestion, larvae hatch in the small intestines and develop into adult worms in the cecum where the anterior end inserts into the colonic mucosa and causes structural changes and localized inflammatory infiltration (37). Children with heavy burden of *Trichuris* can develop colitis similar to inflammatory bowel disease and dysentery syndromes (38, 39). Animal models with chronic trichuriasis have evidence of transmural colonic inflammation and immune profiles dominated by proinflammatory mediators interferon (IFN)-γ, interleukin (IL)-1β, STAT4, tumor necrosis factor (TNF)-α, and IL-6, mimicking both murine models of inflammatory bowel disease and human inflammatory bowel disease (40). Marked elevation of IFN-γ during chronic trichuriasis as well as the production of an IFN-γ homologue by the intestinal helminth have been linked to epithelial dysregulation (41) and progression to chronic *Trichuris* colitis (42). Furthermore, *Trichuris* infection in mice with a genetic predisposition to developing inflammatory bowel disease have accelerated progression of colitis with exaggerated mucosal inflammatory infiltration and cytokine production (43). In addition, clinical studies evaluating children with heavy burden of *Trichuris* infection, indicate colonic pathology with severe, chronic infiltration of inflammatory cells and mucosal destruction consistent with colitis providing supporting evidence that trichuriasis may contribute to human inflammatory bowel disease in parasite-endemic regions (44). Trichuriasis can also be associated with iron-deficiency anemia in children with heavy disease burden resulting from microhemorrhages and blood oozing around the colonic mucosal entry site as seen in *Trichuris* dysentery syndrome (44, 45).

Toxocariasis

Toxocariasis is a zoonotic helminth disease of dogs and cats (*Toxocara canis* and *Toxocara cati*) (46, 47). Humans, most commonly young children, become infected with *Toxocara* spp. after ingesting soil contaminated with *Toxocara* spp. eggs, or rarely when ingesting undercooked meat containing larvae. Following ingestion, L3 larvae hatch from the egg in the intestines and migrate to host tissues including the liver, lungs, heart and central nervous system (CNS) (48). Because humans are accidental hosts, *Toxocara* larvae cannot complete their life cycle in humans and die within host viscera overtime, inducing eosinophilic inflammation and granuloma formation (49). Toxocariasis is typically asymptomatic, but larval migration into host tissues can cause syndromes such as visceral larvae migrans (VLM), neurotoxocariasis (NT) and ocular larvae migrans (OLM).

NT and epilepsy

Cases of acute NT classically present as eosinophilic meningoencephalitis, myelitis, cerebral vasculitis, or seizures. These clinical manifestations are direct results of larval migration and death within the CNS and the sequalae of the profound inflammatory immune response within tissue (50). Specifically, NT-related epilepsy in children and adults is likely due to increased concentrations of pro-inflammatory cytokines within the brain parenchyma, aberrant neurotransmitter activity and parenchymal scarring (51–53). Murine models of chronic NT demonstrate changes in mRNA expression of key pro-inflammatory cytokines (TNF-α and IL-6), inducible nitric oxide synthase, and neurotransmitters (increased norepinephrine and glutamate and decreased GABA, dopamine, and serotonin expression) compared to controls (54–56). Furthermore, *Toxocara* larval migration through tissue induces the formation of eosinophilic granulomas that can cause CNS scarring which become foci of seizure activity. Observed mechanisms of brain injury-measured by increased expression of glial fibrillary acidic protein (GFAP), AβPP, substance P, transforming growth factor β1 (TGF-β1), ubiquitin-proteasome system (UPS), NF-L, S100B, tTG, and p-tau, in mice infected with *Toxocara*–suggest a possible link between NT and chronic neurogenerative (e.g., Alzheimer's) and neuropsychiatric diseases (57–60). Although, adequately powered clinical studies are needed to make definitive conclusions (61, 62).

VLM and asthma

Larval migration through the host lungs is associated with the development of chronic lung disease (63–66). Acute *Toxocara* larval migration and subsequent larval death in the lungs causes wheezing and cough due to airway hyperreactivity and excessive mucous secretion. Beyond acute disease, previous exposure to *Toxocara* is a risk factor for the development of chronic asthma in children (63, 64). *Toxocara* infection in the lungs stimulates innate and type-2 adaptive immune responses, marked by elevated IL-4, IL-5, and IL-13, which results in immunoglobulin class switching to IgE, recruitment and survival of tissue dwelling eosinophils, goblet cell metaplasia and airway hyperreactivity manifesting as asthma. High levels of circulating IgE are capable of binding to mast cells, inciting mast cell degranulation and release of pro-inflammatory mediators further contributing to allergic airway pathology (67–69). Murine models have further demonstrated that *Toxocara* can not only independently cause allergic airway disease but can exacerbate airway inflammation in animals with established allergic airway disease induced by ovalbumin (OVA) sensitization and OVA challenge with overt expression of IL-4, IL-5, and IL-10 (70, 71).

OLM and vision loss

OLM most commonly occurs in older children (classically aged 5-10) and adults as a result of *Toxocara* larval migration and death within the eye. Larval death promotes sustained inflammation causing extracellular matrix remodeling and development of eosinophilic granulomas, leading to a wide

range of ocular disease including chorioretinitis, vitritis, endophthalmitis, and optic neuropathy as well as retinal detachment leading to blindness (72–75). Animal models of OLM demonstrate that matrix metalloproteinases (MMPs), MMP-2 and MMP-9, and prolonged elevated concentrations of pro-inflammatory cytokines IL-6, IL-8, IL-10, and VEGF aid in recruitment of leukocytes, particularly eosinophils, and breakdown fibrin in the posterior chamber extracellular matrix proteins leading to eosinophilic granuloma formation (76, 77). In the U.S. alone, OLM causes approximately 70 cases of blindness annually (78). While reports suggest OLM occurs in 6.6 cases per 100,000 persons, the true global burden of OLM remains unknown (79).

Lymphatic filariasis

LF is mosquito-borne disease caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, though most disease (approximately 90%) is due to *W. bancrofti* (80). Infection occurs when infective stage larvae (L3) are injected into the skin during a mosquito blood meal and travel to the lymphatic vessel where the L3 develop into adult worms (80, 81). Adult worms in the lymphatics release microfilariae into the blood circulation. Pathology is primarily caused by the adult worms, as their presence in the lymphatic vessels can lead to long-term lymphatic inflammation resulting in significant and often disfiguring lymphedema of the lower extremities and other appendages, known as "elephantiasis." Further, over time, filarial lymphedema is often complicated by bacterial infections of the overlying skin and difficulty with ambulation associated with significant morbidity (80, 81).

Additionally, LF can cause other chronic diseases secondary to the microfilaria stage. Microfilariae that transverse the lungs induce a severe allergic airway disease with airway hyperresponsiveness (manifesting as chronic cough and wheezing), pulmonary and peripheral eosinophilia and pulmonary infiltrates, like clinical asthma, known as pulmonary eosinophilia syndrome (82). Pulmonary function tests reveal pulmonary eosinophilia syndrome cause a restrictive lung disease pattern and may lead to pulmonary fibrosis if left untreated. The pathophysiology is thought to be related to release of filarial antigens that have homology to common allergens such as tPE γ-glutamyl transpeptidase. For instance, *B. malayi* infection may lead to γ-GT specific IgG1 and γ-GT specific IgE antibody expansion (83). Murine models of *B. malayi* have demonstrated that pulmonary eosinophilia syndrome is modulated by type-2 cytokines such as IL-4. Interestingly, this disease phenotype can be suppressed by IL-12, leading to decreased IgE, eosinophilia, and airway hyperresponsiveness, thereby downregulating filaria-induced lung immunopathology (84).

Filarial extrapulmonary pathology may additionally involve the joints and the kidneys. Arthritis (and even vasculitis) attributable to lymphatic filariasis is uncommon (mostly reported in Indian patients with *W. brancrofti*) but may manifest either as oligoarthritis or as polyarticular pseudorheumatism (85–88). Symptoms are often unresponsive to non-steroidal anti-inflammatories but improve with anti-filarial treatment (e.g., diethylcarbamazine [DEC]). The pathogenesis is thought to be related to either immune complex deposition or inflammation caused by the presence of the adult worm in the joint space. Regarding the kidneys, patients with filariasis may develop chronic kidney disease manifesting as proteinuria and nephrotic syndrome (89–92). The mechanism is likely driven by immune complex deposition in response to the presence of adult worms and microfilariae. Both the renal tubules and glomeruli may be affected. In an Indian study of 14 patients with filariasis due to *W. bancrofti* and proteinuria, hematuria, or chyluria, six were found to have mesangioproliferative changes, three had inflammatory cell proliferation, and two had endocapillary cell proliferation (93). Immunofluorescence of kidney tissue demonstrated mesangial deposits of IgG alone or in combination with complement 3 (C3) in patients with mesangioproliferative changes and granular deposit of IgG and C3 along the capillary wall in those with endocapillary cell proliferation (93). Another study of patients with filariasis in India due to *B. malayi* indicated that the glomeruli are affected more often than the tubules in symptomatic patients, however noted that proteinuria persisted even after treatment with DEC (94).

Onchocerciasis

The filarial worm *Onchocerca volvulus* is well-known to cause vision impairment, primarily in Africa (though disease foci still exist in Yemen, Venezuela, and Brazil) (95). In 2017, more than 1 million people already had vision loss due to the disease (96). Importantly, intracytoplasmic bacteria (*Wolbachia*) often live symbiotically within *O. volvulus*. The pathology of onchocerciasis is likely due to inflammatory responses against both the microfilariae, which circulate in the subcutaneous tissue and lymphatic system and trigger a host inflammatory response (i.e., granuloma formation and eventually fibrosis) when they die, and symbiotic *Wolbachia*. Repeat infections lead to worse cumulative disease. The specific mechanisms that lead to ocular disease remain unclear. Study of tissue sections have demonstrated infiltrates containing plasma cells, eosinophils, and mast cells (97). Degenerating microfilariae may cause punctate keratitis. Inflammation (and possibly an autoantibody formation) (98, 99) related to microfilariae can lead to anterior uveitis and chorioretinitis. Corneal pathology is associated with increased systemic and corneal type-2 cytokines expression, illustrated by *in vivo* studies in which IL-4 gene knockout mice developed less severe or no *O. volvulus*-mediated keratitis (100). Regarding molecular mimicry, *O. volvulus* antigen Ov39 is cross-reactive with the retinal antigen hr44 and induces ocular

inflammation in rats (99). Interestingly, corneal inflammation is not induced by extracts derived from *O. volvulus* depleted of *Wolbachia* (101), and may be related to expression of adaptor molecules such as TIRAP/Mal (101) and myeloid differentiation factor 88 (102), which is a necessary part of some toll like receptor (TLR) signaling pathways.

O. volvulus may also contribute to a form of incompletely described, but likely progressive epilepsy known as "nodding disease" (103, 104). Nodding disease has been primarily recognized among children in east Africa (particularly Tanzania, Sudan, and Uganda) though outbreaks over the last decade are reported from Uganda, Liberia, Tanzania, the Democratic Republic of Congo, and southern Sudan. Nodding disease classically manifests as episodes during which the head bobs forward repeatedly for several minutes and the individual may seem unresponsive; these episodes are sometimes associated with generalized tonic-clonic and/or absence seizures. Nodding syndrome may progress to significant cognitive disability and, eventually, death (105). No specific cerebrospinal fluid or neuroimaging abnormalities are yet associated with the disease, though some individuals are noted to have significant atrophy of the hippocampal and glia matter (106). The association between *O. volvulus* and nodding disease came about after one Ugandan study suggested a higher rate of epilepsy in communities with higher *O. volvulus* prevalence (107), however, the underlying biological mechanism for such an association remains unclear (108–110). One possibility under active investigation is that nodding syndrome may be due to autoantibodies (e.g., to leiomodin-1) produced in response to *O. volvulus* infection (111).

Trematodes

Schistosomiasis

Three *Schistosoma* species cause most intestinal (*S. japonicum* and *S. mansoni*) and urogenital disease (*S. haematobium*). The host immune response to *Schistosoma* species eggs is responsible for the clinical manifestations of the disease syndromes. Paired adult worms reside in small veins (of the lower urinary tract for *S. haematobium* and of the mesenteric plexus for *S. japonicum*) and release *Schistosoma* species eggs which migrate through associated organs, thereby causing considerable irritation, prolonged inflammation, and granuloma/fibrosis development (112, 113). Schistosomiasis egg migration and the subsequent chronic immune activation can have a wide range of effects on end-organ disease.

Schistosoma-induced renal disease

Renal disease, ranging from asymptomatic to end-stage renal disease (ESRD), is a result of direct egg induced inflammation or *Schistosoma* antigen - immune complex formation within the kidney most commonly seen in *S. mansoni* hepatosplenic disease (114). In a Brazilian longitudinal study of 24 patients with schistosomiasis (likely *S. mansoni* as this is the major species found in Brazil), fifteen (68.1%) had related hepato-splenic disease, thirteen (54.1%) had nephrotic-nephritic syndrome, twenty (83.3%) had hematuria and 18 (75.0%) had hypertension. After nearly 60 months of follow up, nine patients developed ESRD (115). Approximately 5-6% of patients with hepatosplenic schistosomiasis develop glomerular involvement (114). The most common schistosomiasis glomerular disease is mesangial proliferative glomerulonephritis followed by membranoproliferative glomerulonephritis and less commonly focal and segmental glomerulosclerosis, exudative glomerulonephritis and amyloidsis (114, 116). Additionally, urogenital schistosomiasis from *S. haematobium* can cause lower urinary track fibrosis and calcification which can lead to renal outlet obstruction, ureter reflux, interstitial nephritis and ESRD (116).

Schistosomiasis-induced liver disease

S. mansoni's predilection for the venous portal-mesenteric system can result in liver fibrosis, portal hypertension as well as end-stage liver disease. In hepatosplenic schistosomiasis, portal hypertension occurs from an eosinophilic granulomatous reaction to *Schistosoma* egg deposited in presinusoidal portal venules causing presinusoidal hepatic fibrosis typically with preserved liver function (117). Imaging of the liver often shows splenomegaly and calcified eggs along enhanced portal tracks in the liver by CT scan (118). The consequences of portal hypertension include gastric bleeding, such as esophageal varices, which is responsible for an estimated 200,000 deaths annually in sub-Sahara Africa, and ascites (119). Polarized Th2 immune responses during hepatosplenic schistosomiasis not only promotes liver fibrosis but also impairs Th1 anti-viral immunity. Co-infection with HBV and/or HCV, common in schistosomiasis-endemic regions, can accelerate liver pathology particularly advancing viral-induced hepatocellular carcinoma and liver failure (120, 121).

Schistosomiasis-associated pulmonary hypertension (Sch-PH)

Sch-PH is believed to be a leading cause of pulmonary hypertension in *Schistosoma* endemic regions around the world and can result in right-sided heart failure (122). Sch-PH is most commonly associated with chronic hepato-splenic schistosomiasis as a result of *Schistosoma mansoni* infection. Approximately 5-10% of patients with hepato-splenic schistosomiasis will develop Sch-PH which can result in devastating cardiovascular disease including end-stage right ventricular heart failure (123, 124). The immunopathogenesis of Sch-PH is likely multifactorial. Hepato-splenic disease causing portopulmonary hypertension may occur due to the underlying liver disease or from *Schistosoma* egg embolization and inflammation induced vasculopathy with medial thickening,

intimal remodeling and formation of granulomas and fibrosis (125).

Schistosomiasis and malignancy

S. haematobium and *S. japonicum* are both designated biological human carcinogens by the International Agency for Research on Cancer (126).

Urogenital schistosomiasis due to *S. haematobium* is common in endemic areas and complicated by the development of bladder cancer. The incidence of urogenital schistosomiasis-associated squamous cell bladder carcinoma is estimated at 3-4 cases per 100,000 (127). Several mechanisms have been implicated in the oncogenesis of *Schistosoma haematobium* infection (128): 1) *Schistosoma* antigen can increase proliferation and longevity of urothelium cells (129) 2) Elevated p53 levels have been documented in both pre-malignant and malignant lesions associated with schistosomiasis (130, 131) 3) Oncogenic mutation of the KRAS gene can be induced in urothelium exposed to whole parasite extract (132) 4) Soluble *Schistosoma* egg antigens (SEA) increase proliferation and oxidative stress and decrease apoptosis (133) 5) Repeated deposition of eggs in the bladder wall and migration of the eggs through the urothelium results in chronic inflammatory infiltrate (134) and parasite-induced oxygen derived free radicals, genetic mutations and the production of carcinogenic compounds (131, 133, 135) as well as 6) Epigenetic changes *via* hypermethylation of the host genome (136). Of note, *S. haematobium* may contribute to other types of cancer as well. An autopsy study from the Central Pathology Institute of Baghdad, Iraq found that, between 1939-1952, of 2276 autopsies, 174 had carcinoma and 113 had *S. haematobium* involvement of the bladder (137). *S. haematobium* was present in 3 with liver cancer, 7 with bladder cancer, 3 with prostate/genitalia cancer, 2 with intestinal/rectal cancer, and 2 with undescribed cancer types. Particularly in the case of liver cancer, co-infection with *S. haematobium* plus another carcinogenic organism, such as the hepatitis B virus, may amplify *S. haematobium*'s carcinogenic potential (138). Additionally, *S. haematobium* can work in concert with HPV to promote the development of cervical cancer. Proposed mechanisms include *S. haematobium* induced mechanical damage to the cervical epithelium and *S. haematobium* induced local immune modulation creating a niche for HPV proliferation (139, 140).

A less common *Schistosoma* species, *S. japonicum*, is more often associated with liver cancer and colorectal cancer (141), although this is mostly based on epidemiologic associations (142–144) and pathology data (145, 146). *S. japonicum* eggs, retained in the intestinal wall, cause prolonged irritation that can result in fibrosis, mucosal hyperplasia, polyp development, and adenocarcinoma formation (147, 148). Additionally, an "egg embolism" can occur leading to pathology in the liver and other organs (149). A pathology study evaluating the association between *S. japonicum* and liver cancer in 4,611 necropsies revealed 227 cases of hepatocellular carcinoma (HCC); 24 (10.6% of these) were associated with *Schistosoma japonica*. Importantly, 27% of these cases had a positive Hepatitis B surface antigen, indicating the possibility that multiple types of co-infection may synergistically contribute to carcinogenesis (150). *S. japonicum* likely induces multiple mechanisms that contribute to malignant transformation of colonic and/or liver tissue. These mechanisms include chronic inflammation (147, 151), carcinogenic molecules derived from *S. japonicum* itself (152, 153), immunomodulation (154, 155), and oncogenic mutations (156).

Liver flukes

Two trematodes, or liver flukes, are associated with malignancy (*Clonorchis sinensis* and *Opisthorchis viverini* with cholangiocarcinoma) and, similar to schistosomiasis, are classified as biological human carcinogens by the International Agency for Research on Cancer (126, 157, 158). Liver flukes' relative contributions to carcinogenesis are likely related to the length and severity of infection, the host's immune status, and other environmental and host genetic factors (159). Both *Clonorchis sinensis* and *Opisthorchis viverrini* are food-borne trematodes found in East Asia; *C. sinensis* is endemic to southern China, Korea, eastern Russia, and northern Vietnam, whereas *O. viverrini* is endemic to Thailand, Lao People's Democratic Republic, Cambodia, and central Vietnam. Though fewer than 10% of people with liver fluke infections will develop cholangiocarcinoma, the incidence of cholangiocarcinoma is significant in regions of high *C. sinensis* or *O. viverrini* prevalence. For example, the incidence of *O. viverrini*-associated cholangiocarcinoma is approximately 98 per 100,000 people in the highly endemic region of the Thai province of Khon Kaen, where the prevalence of *O. viverrini* infection ranges from 2-70% (160). A Korean study published in 1996 showed that 33% of the cholangiocarcinoma cases evaluated were positive for *C. sinensis* by stool examination (161). Adult flukes can inhabit the biliary track for decades and cause recurrent pyogenic cholangitis. This repeated and/or prolonged inflammation of the biliary tree may contribute to later development of chronic biliary track disease including cholangiocarcinoma, however the exact mechanism(s) by which the liver flukes contribute to carcinogenesis are not yet fully elucidated. Likely liver fluke infection generates multiple mechanisms leading to carcinogenesis, including 1) Mechanical damage from physical contact of the biliary epithelium with the parasite, 2) Inflammatory pathology related to the host-parasite immune response, and 3) Prior to chemical damage from fluke excretory-secretory products (ESPs) (158, 162, 163). A sizeable body of literature exists detailing the carcinogenic potential of ESPs. Fluke ESPs can induce proliferation (164, 165), apoptosis

(166, 167), chromatin remodeling (166), and inflammation of biliary epithelial cells (168). Resulting repetitive insults to the biliary epithelium may lead to hyperplasia and adenomatous changes with subsequent malignant transformation (158).

Paragonimiasis

Paragonimiasis, the lung fluke, results from ingesting metacercariae in raw or undercooked crab or crayfish. The metacercariae encyst in the duodenum, travel through the intestinal wall into the peritoneal cavity, abdominal wall and diaphragm transversing into the lungs and subsequently encapsulating into adult flukes within lung parenchyma (169). During active infection, paragonimiasis can mimic the radiographic appearance and clinical manifestations of pulmonary tuberculosis or even lung cancer, including pleural disease, solitary nodules and cavitary lesions, commonly presenting with chronic cough, chest pain and hemoptysis (170–172). However chronic lung pathology can also occur from pleuropulmonary paragonimiasis. Infection left untreated or repetitive infection with *Paragonimus* spp, most commonly *Paragonimus westermani*, can lead to the development of bronchiectasis and chronic bronchitis (173, 174). The pathophysiology of *Paragonimus* spp. associated chronic lung disease remains unknown. More robust animal models and human clinical studies are needed.

Although rare (occurring in approximately 0.8% cases of paragonimiasis), aberrant migration of *Paragonimus* spp to the brain can also lead to the development of hemorrhagic stroke and epilepsy. In cases of ectopic paragonimiasis, 30-60% occur in the brain and most commonly occurs in children (175). Adult worms migrate through the perivascular connective tissue around the jugular vein and carotid artery into the posterior circulation *via* the skull base foramina leading to mechanical damage secondary to parasitic migratory tracts and formation of eosinophilic granulomas with central necrosis and charcot-leyden crystals (176–178). Early disease typically presents as meningoencephalitis, vasculitis and necrotizing granulomas, manifesting clinically as epilepsy and hemiplegia. Cerebral paragonimiasis can also cause pseudoaneurysms that are often misdiagnosed as noninfectious vascular malformation in children. In 17 patients with cerebral paragonimiasis and hemorrhagic strokes 35% had evidence of pseudoaneurysm and pseudoaneurysm rupture (179). In children with chronic cerebral paragonimiasis, CNS investigation reveals perivascular granulomas formation and calcification and associated cortical and subcortical atrophy (175). In a case series of 14 children with cerebral paragonimiasis, intracranial hemorrhage and eosinophilic granulomas were commonly identified. Despite targeted therapy, two of the 14 children had persistent hemiplegia on long-term follow-up secondary to sequelae of hemorrhagic strokes (180). In addition to epilepsy and paralysis, children with cerebral paragonimiasis can also have long-term behavioral changes. If treated early with antiparasitic therapy, seizures generally improve. However, dizziness, memory loss, personality changes, and loss of fine motor function, often do not completely resolve (181).

Cestodes

Neurocysticercosis (*Taenia solium*)

Taenia solium (the pork tapeworm) can cause intestinal disease ("taeniasis"), which is acquired after ingestion of *T. solium* larvae ("cysticerci") *via* infected pork or cysticercosis, due to inadvertent consumption of *T. solium* eggs *via* exposure to a person or pig with taeniasis (182, 183). Cysticercosis can develop once *T. solium* eggs hatch into larvae in the intestine, migrate to various host organ systems, and develop into cystic larvae within the tissue. Neurocysticercosis (NCC) is the most consequential form of cysticercosis and occurs when cysticerci develop in the CNS (including the brain, eyes, and spinal cord). NCC is a leading cause of adult-onset epilepsy worldwide (184). Most of the clinical manifestations seen in NCC are related to the host immune response to the parasite, and are dependent on the number, stage, and size of cysticerci. For instance, nearly 80% of patients diagnosed with NCC report at least one lifetime seizure (185). Seizures typically are seen in patients who have parenchymal NCC (186). They are triggered by disruption of the brain parenchyma and therefore neuronal signaling caused by certain stages of *T. solium* cysts, specifically as cysts naturally progress from viable to a final calcified stage (187). Viable cysts are often able to evade the immune response (187–189), thus it is the inflammatory reaction to degenerating cysts (which have lost their ability to modulate the host immune system) and mechanical obstruction of normal neuronal pathways caused by residual calcifications from old cystic lesions that provoke seizures (190). Further, the degree of cyst-induced inflammation may be directly related to frequency of seizure recurrence (191). Studies of human brain tissue sections with NCC suggested that the initial immune response (prior to peri-cyst granuloma formation) is characterized by innate and Th1 cells and cytokines, including natural killer cells, macrophages, T cells, and interleukin (IL)-12 (187). Subsequently, as granulomas develop around degenerating cysts, a chronic immune reaction develops, characterized by both type-1 and type-2 immune responses (192). Additionally, symptomatic NCC patients may produce lymphocytes primed towards Th1 responses (193) and have distinct TLR polymorphisms (194). Calcified NCC specifically has been associated with not only unique TLRs but also higher serum levels of matrix metalloproteinases (MMP)-9; the same study found MMP-9 to be associated with seizure recurrence (195).

While most patients diagnosed with NCC have parenchymal disease (and calcified parenchymal disease is more common than viable parenchymal cysts), a rare but important subset of NCC is extra-parenchymal disease (186).

Extra-parenchymal disease is defined by cystic lesions that develop in the ventricles, subarachnoid space, spine, or retina. Ventricle and subarachnoid cysts can cause symptoms *via* mass effect on surrounding tissues, the host inflammatory response to the cyst tissue can trigger aseptic meningitis, and both the cysts themselves and scarring from accompanying inflammation can cause obstructive and/or communicating hydrocephalus that often requires invasive intervention *via* placement of a ventriculo-peritoneal shunt or surgical removal of cysts (196). Cerebrospinal fluid from patients with subarachnoid disease seem to have significantly elevated levels of type-1 and type-2 cytokines compared to patients with parenchymal disease (197, 198).

Echinococcosis

Cystic echinococcosis (*E. granulosus*) and alveolar echinococcosis (*E. multilocularis*) results from ingestion of *Echinococcus* spp eggs in contaminated soil (199–201). From the intestines the eggs hatch releasing oncospheres that penetrate the intestinal wall and travel classically to the liver or lungs and, aberrantly, to other organs like the brain where they develop into thin-walled cysts. Patients with *Echinococcus*

TABLE 1 Proposed mechanisms of helminth induced non-communicable diseases.

Helminths	Non-communicable disease	Proposed Mechanism of disease	References
Nematodes			
Ascaris spp.	Larval migratory stage: anemia, asthma, chronic obstructive pulmonary disease Adult intestinal stage: anemia, biliary stenosis, end-stage liver disease	-Chemical damage from excretory secretory products (ESPs) with immunogenic proteins during larval migration -Mechanical damage from direct larval migration -Adult intestinal worm obstructing the biliary tract	(15, 18, 20, 23, 25, 214)
Hookworm (*Ancylostoma duodenale*; *Necator americanus*)	Larval migratory stage: chronic obstructive pulmonary disease Adult intestinal stage: anemia	-Chemical damage from ESPs with immunogenic proteins during larval migration -Mechanical damage from direct larval migration -Adult intestinal worm extravasation of blood	(30–34)
Trichuris trichiura	Adult intestinal stage: inflammatory bowel disease, anemia	-Insertion of anterior end into intestinal mucosa inducing local inflammation	(40, 41, 43, 45, 215)
Toxocara cani/cati	Larval migratory stage: Asthma, epilepsy, neurodegenerative diseases, neurobehavioral diseases, vision loss/blindness	-Larval migration and death in viscera causing eosinophilic infiltration and granulomas	(47, 51–53, 59, 61, 64, 68, 70, 75, 76, 216)
Anisakis simplex	Asthma, urticaria	-Cross-reactivity of *A. simplex* antigen with common allergens -High levels of *A. simplex* specific IgE	(217–219)
Lymphatic Filariasis (Wuchereria bancrofti, Brugia malayi, Brugia timori)	Lymphangitis and resulting complications related to chronic lymphedema (e.g., elephantiasis) and/or urogenital disease including kidney disease; less commonly, arthritis Microfilariae: pulmonary eosinophilia syndrome, renal disease	-Adult worms cause inflammation in the afferent lymphatic channels, leading to chronic obstructive changes -Renal disease likely due to immune complex deposition, among other mechanisms -Lung disease due to filarial antigens that have homology to common allergens, IL-4 mediated	(81, 83, 86, 87, 94)
Loa loa	Chronic kidney disease; less commonly, arthritis	-Etiology not completely elucidated	(88, 88, 220, 221)
Onchocerca volvulus	Vision impairment and dermatologic disease, progressive epilepsy	-Tissue damage due to inflammatory response to microfilariae and *Wolbachia*; an additional autoimmune component is suspected	(98–100, 107, 108, 110)
Trematodes			
Schistosoma	Malignancy (bladder, liver, colon, cervical), pulmonary hypertension and end-stage heart disease, portal hypertension, chronic genito-urinary track disease (e.g.,	-*Oncogenic mutations in* p53 and KRAS the urothelium	(114, 117,

(Continued)

TABLE 1 Continued

Helminths	Non-communicable disease	Proposed Mechanism of disease	References
	glomerular damage leading to hypertension, nephritis, nephrotic syndrome, end-stage renal disease)	-*Schistosoma egg* antigen increases proliferation and longevity of host cells. -*Schistosoma egg* induce chronic inflammation, fibrosis, granuloma formation	122, 129, 130, 132, 141, 142, 144, 222)
Clonorchis *Opisthorchis*	Malignancy (cholangiocarcinoma)	-Mechanical damage from physical contact of the biliary epithelium with the parasite -Inflammatory pathology related to the host-parasite immune response, and chemical damage from fluke ESPs.	(158, 161, 163, 166, 167)
Paragonimus	-Bronchiectasis, chronic bronchitis -Epilepsy, stroke	-mechanical damage secondary to parasitic migratory tracts and eosinophilic granulomas	(174, 176–178)
Cestodes			
Taenia solium	When manifested as neurocysticercosis: epilepsy, obstructive and/or communicating hydrocephalus	-Host immune response to degenerating cysts in the central nervous system -Mechanical obstruction of normal neuronal pathways caused by residual calcifications from old cystic lesions	(184, 185, 187, 192, 197)
Echinococcus	-Hepatobiliary: biliary track fistulas, biliary cirrhosis, cholangitis, pancreatitis, and portal hypertension with associated gastrointestinal bleeding -Pulmonary: asthma -Cerebral: epilepsy, stroke -Renal: tubulointerstial nephritis, glomerulonephritis (minimal change disease, mesangioproliferative) and nephrotic syndromes.	-Mass effect from hepatic cysts causes increased pressure on hepatic and biliary tissue -Acute rupture of pulmonary cysts, release of immunogenic antigens from the cystic fluid, and exaggerated Th2 immune response -Cytotoxic effects on the surrounding cerebral parenchyma resulting in inflammatory recruitment and tissue necrosis -Immune-mediated	(201, 206, 210, 223)

cysts are typically asymptomatic until the lesions create a mass effect on surrounding tissue or rupture causing a systemic inflammatory response (201). The clinical course of echinococcosis in different organ compartments is associated with a wide spectrum of complications that can lead to chronic disease in the liver, lungs, brain and kidneys (201, 202). In a cohort of 506 patients with cystic echinococcosis, 204 developed long-term complications as a result of their illness (202).

Patients with liver lesions, from either Cystic *Echinococcus* or Alveolar *Echinococcus*, have high risk of hepatic complications. Approximately 1/3rd of all patients with hepatic disease will develop a long-term complication of the hepatobiliary system including biliary track fistulas, biliary cirrhosis, cholangitis, pancreatitis and portal hypertension with associated gastrointestinal bleeding (201, 203). Mass effect from hepatic cysts cause increased pressure on hepatic and biliary tissue leading to compression and necrosis of adjacent tissues. Bile duct damage, rupture and subsequent development of biliary fistulas are thus common. The mass effect of hepatic cysts can also reduce portal vein inflow causing portal vein thrombosis and portal hypertension as well as compression and displacement of hepatic veins leading to Budd-Chiari syndrome. Liver pathology in patients with *E. multilocularis* hepatobiliary cysts were found to have periportal fibrosis, perilobular fibrosis and amyloid deposition (204). Pulmonary cysts can also cause mass effect on surrounding mediastinal structures, however clinical manifestations most commonly result from acute rupture of pulmonary cysts, release of immunogenic antigens from the cystic fluid, and exaggerated type-2 immune responses leading to an asthma-like syndrome of wheezing and coughing (201). Furthermore, some mouse models have shown that the presence of *Echinococcus* antigens may exacerbate established allergic airway disease through increased type-2 cytokine signaling resulting in histopathologic changes consistent with asthmatic disease (205). Although rare, occurring in approximately 1% of all cases of *E. multilocularis*, cerebral echinococcosis is associated with high mortality, with a 10-year survival rate of 28.4%. The presence of *E. multilocularis* cystic fluid can have cytotoxic effects on the surrounding

cerebral parenchyma resulting in inflammatory recruitment and tissue necrosis (206). Of those with cerebral echinococcosis that survive, nearly 30% will have long-term neurologic sequalae including epilepsy, vision loss and hemiplegia (207–209). Several case reports and case series suggest that echinococcosis from either *E. granulosus* or *E. multilocularis* can be associated with kidney disease. The mechanism of *Echinococcus* renal disease is thought to be immune-complex mediated leading to tubulointerstial nephritis, glomerulonephritis (minimal change disease, mesangioproliferative) and nephrotic syndromes (210). In a case series of patients with hepatic cysts with associated proteinuria, kidney biopsy demonstrated disease was driven by hydatid antigen and the development of immune-mediated glomerulonephritis (211–213). As the literature related to extra-hepatic echinococcosis is limited to case reports and cases series, more in depth studies are needed to determine the mechanisms of *Echinococcus* driven extra-hepatic chronic diseases.

Conclusions

Helminths, which disproportionately affect persons living in poverty within LMICs, represent a major driver of morbidity on a global scale. Their role in driving NCDs including chronic lung disease, cancer, cardiovascular disease and inflammatory bowel disease are plausible but more robust mechanistic studies and human clinical trials are required to draw definitive conclusions (Table 1). These studies are necessary to aid in uncovering risk factors associated with the rising incidence and prevalence associated with NCDs in LMIC and other helminth-endemic regions around the world. Understanding this connection between helminths and NCDs will aid in targeted public health programs to reduce helminth-induced NCDs and reduce the high rates of morbidity in these regions. In the mean-time focus on helminth prevention and control efforts are critical. Access to mass drug treatment programs, water, sanitation, and hygiene interventions and health education programs for high-risk populations, especially children and pregnant persons, will result in reduced worm burdens and subsequently reduced morbidity. Elimination efforts should also remain a public health priority as the complex mechanisms of helminth induced NCDs are being evaluated.

Author contributions

YW and JW conceptualized, wrote and edited the manuscript. MD, SA, CS-R, and EC wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by NIH NIAID K23 K23AI168583-01 and NIH NIAID K08 AI143968-01.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 15 September 2022
DOI 10.3389/fimmu.2022.938011

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 06 May 2022
ACCEPTED 22 August 2022
PUBLISHED 15 September 2022

CITATION
Rivera-Correa J and Rodriguez A (2022) Autoantibodies during infectious diseases: Lessons from malaria applied to COVID-19 and other infections.
Front. Immunol. 13:938011.
doi: 10.3389/fimmu.2022.938011

Autoantibodies during infectious diseases: Lessons from malaria applied to COVID-19 and other infections

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Autoimmunity is a common phenomenon reported in many globally relevant infections, including malaria and COVID-19. These and other highly inflammatory diseases have been associated with the presence of autoantibodies. The role that these autoantibodies play during infection has been an emerging topic of interest. The vast numbers of studies reporting a range of autoantibodies targeting cellular antigens, such as dsDNA and lipids, but also immune molecules, such as cytokines, during malaria, COVID-19 and other infections, underscore the importance that autoimmunity can play during infection. During both malaria and COVID-19, the presence of autoantibodies has been correlated with associated pathologies such as malarial anemia and severe COVID-19. Additionally, high levels of Atypical/Autoimmune B cells (ABCs and atypical B cells) have been observed in both diseases. The growing literature of autoimmune B cells, age-associated B cells and atypical B cells in Systemic Lupus erythematosus (SLE) and other autoimmune disorders has identified recent mechanistic and cellular targets that could explain the development of autoantibodies during infection. These new findings establish a link between immune responses during infection and autoimmune disorders, highlighting shared mechanistic insights. In this review, we focus on the recent evidence of autoantibody generation during malaria and other infectious diseases and their potential pathological role, exploring possible mechanisms that may explain the development of autoimmunity during infections.

KEYWORDS

autoantibodies, malaria, atypical B cells, COVID-19, autoimmunity

Autoantibodies during infection

Autoantibodies are well-known mediators of pathology in autoimmune disorders, such as Systemic Lupus erythematosus (SLE) where they cause organ inflammation and damage (1). However, it has been less appreciated that autoimmunity is a common phenomenon during infections, including viral, bacterial and parasitic diseases such as HIV, tuberculosis or malaria, among others (2, 3). Many human infections with a highly inflammatory component, such as malaria and COVID-19, have been associated with high levels of autoantibodies targeting various host molecules, such nucleic acids (DNA, RNA), membrane proteins, carbohydrates, and phospholipids (such as phosphatidylserine (PS) (2, 3). Although infection-induced autoantibodies have been reported for a long time, only recently a pathogenic role during infection has been identified. For example, infection-induced autoantibody production has been associated with transient or post-infection pathologies, such as malarial anemia (4–8) Intriguingly, most of these autoantibodies are present at higher levels during acutely infected patients and drop upon treatment or resolution of infection (2). The regulation and roles of these autoantibodies are not completely understood.

ABCs and atypical B cells are known to expand and secrete autoantibodies in autoimmune disorders, such as SLE (9, 10). ABCs were largely described in mouse studies to accumulate with age, in autoimmune disease models and during acute viral infections and were delineated by expression of the non-classical B cell markers CD11c and T-bet (10). On the other hand, atypical B cells were initially described as a memory population that accumulate in patients with HIV, tuberculosis, repeated malaria exposures and other infections, as well as in autoimmune patients, and were mainly characterized by the lack of expression of CD27 and CD21, but also by expression of CD11c and T-bet (11, 12). During malaria, these atypical B cells have been implicated in secreting autoimmune antibodies against PS, and in contributing to malarial anemia (13, 14). ABCs and atypical B cells represent a heterogonous population with many different development origins, that could include both classical and non-classical routes, such as the extrafollicular (EF) route which has been associated with autoantibody secretion. Polyclonal activation and relaxation of B cell tolerance during complex infectious diseases, could account for this autoimmune phenomenon (15–17). Due to the variable nomenclature used in the literature to describe these cells in different settings, we will use "ABCs and atypical B cells" as a generalized term to describe Atypical/Autoimmune/Age associated B cells and Double Negative cells (DNs). Recently, ABCs and atypical B cells have been implicated in the autoimmune phenomena during infection, as they were shown to expand and secrete autoantibodies in autoimmune disorders, such as SLE. Concurrently, ABCs and atypical B cells have also been reported to expand in acutely infected patients with malaria (7, 8, 18), HIV ((11, 19)), COVID-19 ((20–23)) and other infections, as well as to accumulate in malaria-experienced individuals with repeated seasonal exposure (Portugal et al., 2017).In this review, we will summarize the evidence reinforcing the relationship between autoantibodies and pathology as well as possible mechanisms explaining its appearance. We will focus on malaria patients but will also expand to recent evidence in COVID-19 patients and other infections.

Pathological role of autoantibodies during malarial anemia and other complications

Autoantibodies during both human and mouse malaria have been reported for decades, but until recently they had not been functionally studied. Malaria leads to production of a range of autoantibodies targeting all kinds of host molecules and cells, such as the phospholipid PS (2, 24, 25). Autoantibodies targeting PS have been directly shown to promote malarial anemia in rodent infections with *P. yoelii* through binding to PS on uninfected red blood cells (RBCs) and promoting their premature clearance (4). In this mouse model, young RBCs (reticulocytes) were preferentially targeted by these anti-PS antibodies, prolonging anemia recovery. These findings were validated in *P. falciparum*-infected patients which showed an inverse correlation between anti-PS antibodies and hemoglobin levels in different cohorts, including French travelers with post malarial anemia (4), acutely infected German travelers (7) and Ugandan pediatric patients with complicated *P. falciparum* malaria (6). Additionally, anti-PS antibodies were also corelated with anemia in a cohort of Colombian patients suffering from either *P. falciparum* or *P. vivax* malaria (Rivera-Correa et al., 2020), highlighting the presence of this correlation in different cohorts around the world. Lastly, these results were also expanded to four of the human *Plasmodium* species as evidenced by a study in Malaysia showing significant levels of anti-PS antibodies in *P. vivax*, *P. falciparum*, *P. malariae* and *P. knowlesi* malaria (5). In this cohort, anti-PS were associated with early anemia in *P. vivax* and *P. falciparum*-infected patients. In addition to anti-PS antibodies, *P. vivax*-malarial anemia has also been correlated with other autoantibodies targeting RBC surface proteins such as spectrin and band 3 in a Brazilian cohort (26, 27). Altogether, these studies show the strong relationship of anti-PS and other autoantibodies with malarial anemia in different human malaria cohorts around the world.

In addition to anemia, autoantibodies have been hypothesized to promote other malaria-associated pathologies (25). In a study of a pediatric Ugandan cohort suffering from severe *P. falciparum* malaria, anti-PS antibodies and anti-dsDNA antibodies were associated with acute kidney-injury (AKI), post-discharge mortality and morbidity, in addition to anemia (6). The mechanism involved in promoting other malaria-associated

pathologies is not well understood. Previous studies on mouse models of malaria have linked dsDNA autoantibodies in promoting kidney pathology through the accumulation of immune complexes (28). Despite multiple *Plasmodium* species being reported to lead to kidney pathology, the relationship between kidney immune complex deposition and kidney pathology has only been reported in *P. malariae* malaria (29). This suggest that other non-autoantibody immune factors are needed in addition to promote kidney pathology in other human malarias underscoring the complexity of this phenomena (30). Lastly, there have also been several studies reporting autoantibodies against brain-associated antigens in patients suffering from *P. falciparum* cerebral malaria, but their pathogenic relevance remains to be determined (25). Although the pathogenic role of autoantibodies during malaria has been more evident, a possible protective role against infection cannot be disregarded. Specific autoantibodies have been correlated with protection against severe malaria (31, 32). Moreover, reports have shown that sera from patients with autoimmune diseases can bind to the parasite and inhibit parasite growth *in vitro* (33, 34). These findings suggest that autoantibodies could have divergent roles as both protective and pathogenic during malarial infection. These divergent roles have been comprehensibly reviewed in previous publications (2, 24, 25).

Possible mechanisms leading to autoantibody production during malaria

The generation of autoimmunity involves a complex mix of genetic and environmental factors that are not well understood in autoimmune disorders and much less in infection. Epitope spreading, bystander activation, molecular mimicry, and cryptic epitopes are particular phenomena that explain the generation of specific autoantibodies during some infections, however, they do not seem to explain the broad variety of self-antigen targets observed in complex infections such as malaria (2) and COVID-19. To add to this complexity, malaria parasites are eukaryotic parasites that share many similar antigenic targets, such as PS and dsDNA, that could target both the parasite and the host cells equally. Additionally, hemolysis during malaria and other infections, exposes many host antigens, such as PS, that can activate the immune system and activate polyclonal B cell responses (35).

Autoantibodies are secreted by autoreactive B cells that get activated by a combination of specific signals that do not normally occur in non-pathogenic immune responses. A body of research, mainly in the SLE field, has described a specific B cell subset that is expanded and is able to secrete autoantibodies during autoimmune disorders called Age/Autoimmune B cells (ABCs and atypical B cells) in mice and a similar population called Double Negative B cells (DNs) in humans (9, 17). A similar population has been reported to be expanded during malaria (11) and in highly inflammatory infections such as HIV, tuberculosis and COVID-19. These cells differ from other B cell populations in the expression of characteristic markers such as low classical memory B cell markers CD21 and CD27, high expression of transcription factor T-bet and integrin CD11c as well as surface expression of FcRL5. Additionally, these cells have been reported to express chemokine receptors such as CXCR3 and other markers. A summary of the nomenclature and markers described for this population has been summarized in previous reviews (36, 37). Further evidence that ABCs and atypical B cells generated during infection and autoimmune disorders are closely related comes from scRNAseq analysis of these cells in malaria patients, which share similar transcriptional profiles with HIV patients, but also with patients from different autoimmune disorders (SLE, rheumatoid arthritis or common variable immunodeficiency), suggesting they share common drivers of expansion and function (38).

During malaria, studies have reported the refractoriness of human atypical B cells to secrete antibodies *in vitro* and reduced B cell receptor (BCR) signaling in response to soluble antigens (18), but atypical B cells were responsive to membrane-bound antigens (39). Additionally, indirect evidence implicates that atypical B cells secrete anti-malaria antibodies, suggesting divergent roles for these cells during malaria (36, 40).

Accordingly, a recent study reported the presence of malaria-specific atypical B cells and found that atypical B cells could secrete antibodies with T cell help (41). Lastly, atypical B cells proliferate in response to malaria, but also to vaccination, indicating that they are part of a wider alternative lineage of B cells that is a normal component of healthy immune responses (42). Additional studies have directly shown that ABCs and atypical B cells secrete autoantibodies (14). Accordingly, a report focused on studying the repertoire of ABCs and atypical B cells in malaria-experienced individuals revealed enrichment of VDJ gene usage associated inherently with autoreactivity (V_H4-34) (38). Altogether these data suggest that ABCs and atypical B cells could be major secretors of pathogenic autoantibodies, such as anti-PS. These studies highlight the complexity of ABCs and atypical B cells and their possibly divergent roles in both protective and pathogenic responses during malaria (43).

Recent insights into the mechanisms leading to autoreactive B cell generation have been elucidated in different mouse models. In autoimmune settings, the integration of primarily three signals are needed to generate ABCs and atypical B cells: BCR signaling, specific type 1 cytokines (IL-21 and/or IFNγ) and nucleic acid sensing toll-like receptors (TLR7 and TLR9) (9, 10, 44). During malaria, the integration of BCR signaling, IFNγ and TLR9 were deemed essential for ABCs and atypical B cells expansion and anti-PS autoimmunity during mouse *P. yoelii* infection (13). Similar findings were published in *P. falciparum* malaria patients, describing how IFNγ and TLRs were important for

ABCs and atypical B cells generation (11, 12, 45). A recent study also expanded the role of IFNγ in promoting an ABCs/Atypical B cell phenotype in *P. vivax* patients (46). In the SLE field, the integration of these cytokines and TLRs, have been attributed to promote an alternative B cell differentiation pathway titled the extrafollicular (EF) pathway (47). In contrast to the classical germinal center (GC) pathway that gives rise to long-lived memory B cells or plasma cells, the EF route through polyclonal activation gives rise to short-lived plasmablasts/plasma cells (PB/PCs) (16, 17). The EF route is considered the main route by which DNs (analogs of ABCs and atypical B cells, named for being $CD27^{-}IgD^{-}$) can arise and secrete autoantibodies in SLE patients. Polyclonal B cell activation has been a hallmark during malaria and contributes to the B cells dysfunction observed during the disease (48). Accordingly, a study on mouse *P. yoelii* malaria, revealed that hemolysis-induced PS polyclonal activation of B cells, through PS-receptor AXL, accounted for great part of the polyclonal responses that lead to the accumulation of short lived PB/PCs that secrete non-specific antibodies and limit protective humoral immunity (35). In this study, they also reported that blocking PS exposure limits non-specific polyclonal PB/PC expansion and reduces *P. yoelii* infection in mice. Moreover, this accumulation of short-lived PB/PCs dampens the essential GC response needed for proper anti-malarial antibody responses (49). Additionally, the extension of time by which this polyclonal activation of B cells is prolonged could account for reports of sustained autoantibodies post-infection (for at least 1 month in *P. vivax* and *P. falciparum* infections) that may contribute to associated pathologies, such as post-malarial anemia and increased hospital post-discharge mortality (4, 24) (6). Altogether, these data suggest that these mechanisms, that are possibly shared between SLE and malaria, could explain the activation of the pathogenic autoantibody responses we see during acute malarial infection.

Lessons from malaria applied to COVID-19 and other infections

Other highly inflammatory infections have been associated with generation of autoantibodies, most recently noted in the ongoing COVID-19 pandemic (3, 50). Similar to malaria, high levels of circulating autoantibodies have been reported in COVID-19 patients, but surprisingly, circulating immune complexes were not increased in these patients. The autoantibodies include similar targets to malaria, such as PS and dsDNA (51), but also expand to newer targets such Annexin A2 (52), that have not been studied in malaria patients. The autoantibody repertoire seems to be broad during COVID-19 and is not clear if it's selected preferably against any autoantigen although their pathogenic role has been highly suggested by multiple studies (53, 54). The malaria-shared autoantibodies anti-PS and anti-dsDNA have been correlated with severity in COVID-19-patients (51), similarly as they have in malaria (Table 1). Autoimmune anti-Annexin A2 also correlated with mortality (52). A study in mice, reported that a range of anti-phospholipid antibodies could promote the pathological coagulation defects highly associated with COVID-19 infections (55). Moreover, the number of antibodies found targeting immune molecules such as cytokines (ex. Type I interferon) has been a highly reported phenomenon in severe COVID-19 (57–59). Accordingly, malaria has also been associated with targeting of immune components such as *IFNGR2* (60), which could distinguish malaria from bacterial blood infection in a small cohort of Ghanese children. Furthermore, early detection of a set of autoantibodies that included anti-IFN-a2, and five anti-nuclear autoantibodies (ANAs) (Ro/SS-A, La/SS-B, U1-snRNP, Jo-1, and P1) that are also commonly associated with (SLE), could anticipate distinct patterns of the puzzling phenomenon of Post-acute sequelae of COVID-19 (PASC) or "Long-COVID" (56). These reports suggest pathological implications of a range of autoantibodies in COVID-19 patients both at acute and post-infection manifestations. The mechanisms that give rise to autoantibodies during COVID-19 could be similar to the ones in malaria and SLE patients (61). Various studies have reported the expansion of ABCs and atypical B cells (20, 62), a phenomenon that could be explained by the enhanced EF route of B cells reported in COVID-19 patients (63). Additionally, the "relaxation" of B cell tolerance has been suggested as an additional mechanism promoting autoreactive B cells and autoantibody secretion in COVID-19 patients (64).

Many other infections also lead to similar autoantibodies as observed during malaria (2). Different anti-phospholipid antibodies have been reported during infections of important global pathogens such as *Mycobacterium tuberculosis* (65), HIV (66) and others such as hepatitis C, cytomegalovirus, varicella zoster, Epstein-Barr virus, adenovirus, and parvovirus B (67, 68). Active tuberculosis has been associated with a range of autoantibodies including with high levels of anti-cardiolipin and other anti-phospholipid antibodies (65, 69). Additionally, autoantibodies targeting RBC components have been reported to increase tuberculosis susceptibility in HIV patients through erythrophagocytosis (70), suggesting a pathogenic role for autoantibodies in co-infection scenarios. Furthermore, anti-phospholipid antibodies, such as anti-cardiolipin, have been utilized as a diagnostic tool for active *Treponema pallidum* infections, being one of the primary methods to diagnose syphilis in humans (71). A similar application was recently suggested for Lyme disease diagnosis (72). Anti-phospholipid and anti-ganglioside autoantibodies were also reported to be correlated in Zika virus-associated Guillain-Barré syndrome patients from Brazil (73, 74). Moreover, autoimmunity against PS and ABCs and Atypical B cell expansion was reported to delay anemia recovery in mice infected with African trypanosome *Trypanosoma brucei* (75). These findings were translated to a cohort of Ugandan Human African

TABLE 1 Autoantigen targets for autoantibodies and their associated pathology during malaria or COVID-19. n/d, not determined.

Self-antigen	Malaria	Associated with Atypicals or ABCs?	COVID-19	References
Phosphatidylserine (PS) and/or other Phospholipids(PL)	Anemia (*P. falciparum and P. vivax*) Acute kidney injury (AKI) (*P. falciparum*) Mortality and morbidity (*P. falciparum*) Complicated malaria (*P. vivax*)	Yes	Severity Coagulation defects (general αPL)	(4–8, 51, 55)
Double stranded-DNA (dsDNA)	Anemia (*P. falciparum*) Acute kidney injury (AKI) (*P. falciparum*)	Yes	Severity	(6, 7, 51)
Red Blood Cell whole lysates or specific protein antigens (ex. Band 3 and Spectrin)	Anemia (*P. falciparum* and *P. vivax*) Complicated malaria (*P. vivax*)	Yes	Severity	(6–8, 25, 26)
Annexin A2	n/d	n/d	Severity Mortality	(52)
Other autoantibodies	Cerebral malaria	n/d	Severity Mortality Long-COVID	(3, 25, 53, 54, 56)

trypanosomiasis (HAT) patients where anti-PS levels were elevated in acutely infected patients. Similarly to COVID-19 and malaria, cytokines can be autoantibody targets during different infections such as: type II IFN during infections with intra-macrophagic microbes, IL-17A/F during mucocutaneous candidiasis and IL-6 during staphylococcal diseases (76). Since the signals leading to ABCs/Atypical B cell expansion, such as TLR ligation and IFN-γ, are present in many other infections, these cells are proposed to be a possible common source for autoantibodies during different infections (11, 15). Altogether,

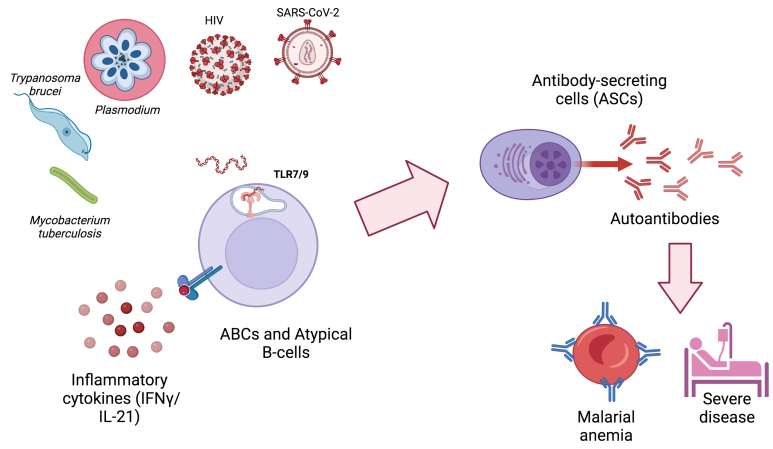

FIGURE 1
ABCs and atypical B cells are generated during different infections and secrete autoimmune antibodies that can contribute to pathology. ABCs and atypical B cells generation during infections requires at least two signals: 1) the activation of TLR9 or TLR7, typically by nucleic acids derived from the infectious agent or by host mitochondrial DNA released by neutrophil extracellular traps (NETs); 2) exposure to IFN-γ and/or IL-21. Autoimmune antibodies generated by autoimmune antibody-secreting cells (ASCs) can contribute to pathology and severe disease. Created with BioRender.com.

these data suggest a global pathogenic role of autoantibodies during malaria, COVID-19 and other infections with shared auto-antigen targets and related mechanisms of pathogenesis.

Conclusions

High levels of autoantibodies are observed in many relevant infections, such as malaria and COVID-19. Their presence during infection has been reported extensively, but their contribution to pathology has been a recent research focus. The growing literature on the activation and expansion of ABCs and atypical B cells in autoimmune disorders such as SLE, has contributed with mechanistic insights that may be relevant for the generation of autoantibodies during malaria, COVID-19 and other infections.

Specifically in malaria, autoantibodies contribute to pathogenesis through the binding of anti-PS autoantibodies to uninfected erythrocytes, promoting malarial anemia. The expansion of ABCs and atypical B cells and their ability to secrete anti-PS and other autoantibodies in both mouse and human malaria marks them as a primary candidate responsible for the generation of autoimmunity during infection. The signals driving ABCs and Atypical B cell expansion, such as TLR ligation, are present in many autoimmune disorders as well as infections, suggesting shared mechanistic pathways for autoreactivity in scenarios as diverse as SLE, malaria and COVID-19 (Figure 1). However, the transient aspect of infection-induced autoantibodies and the heterogeneity of ABCs and atypical B cells and their divergent functional roles during malaria highlight the complexity of this phenomenon during infection. Further studies are needed to understand the mechanisms by which these cells arise, explore fully their different roles and explain their dynamics during malaria and other infections.

Author contributions

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

Funding

AR was supported by NIH grant R21AI151349.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 16 September 2022
DOI 10.3389/fimmu.2022.963723

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 07 June 2022
ACCEPTED 15 August 2022
PUBLISHED 16 September 2022

CITATION
Sardinha-Silva A, Alves-Ferreira EVC and Grigg ME (2022) Intestinal immune responses to commensal and pathogenic protozoa.
Front. Immunol. 13:963723.
doi: 10.3389/fimmu.2022.963723

Intestinal immune responses to commensal and pathogenic protozoa

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The physical barrier of the intestine and associated mucosal immunity maintains a delicate homeostatic balance between the host and the external environment by regulating immune responses to commensals, as well as functioning as the first line of defense against pathogenic microorganisms. Understanding the orchestration and characteristics of the intestinal mucosal immune response during commensal or pathological conditions may provide novel insights into the mechanisms underlying microbe-induced immunological tolerance, protection, and/or pathogenesis. Over the last decade, our knowledge about the interface between the host intestinal mucosa and the gut microbiome has been dominated by studies focused on bacterial communities, helminth parasites, and intestinal viruses. In contrast, specifically how commensal and pathogenic protozoa regulate intestinal immunity is less well studied. In this review, we provide an overview of mucosal immune responses induced by intestinal protozoa, with a major focus on the role of different cell types and immune mediators triggered by commensal (*Blastocystis* spp. and *Tritrichomonas* spp.) and pathogenic (*Toxoplasma gondii*, *Giardia intestinalis*, *Cryptosporidium parvum*) protozoa. We will discuss how these various protozoa modulate innate and adaptive immune responses induced in experimental models of infection that benefit or harm the host.

KEYWORDS

intestinal immunity, commensal, protozoa, blastocystis, *Cryptosporidium*, *Giardia*, *Toxoplasma*

Introduction

Mucosal tissue is a physical barrier composed of biochemical and immunological components at the interface between the host and the external environment. Mucosal immunity plays a fundamental role in promoting tolerogenic immune responses in order to maintain homeostasis in addition to providing the first line of defense against pathogenic and non-pathogenic microorganisms (1).

The gastrointestinal tract is the largest mucosal tissue in the human body, which harbors a diverse community of commensals including prokaryotic bacteria, eukaryotic fungi, and protozoa, as well as other organisms such as intestinal viruses, helminth parasites, and pathogenic protozoa (Figure 1). Although commensals are commonly referred to as symbiotic microorganisms that are either non-pathogenic or beneficial (referred to as mutualists) to their host (2), it is increasingly evident that their presence plays an important role in reshaping the host immune system (3). Beyond their role in digestion and nutrient acquisition, the presence of commensal organisms is fundamental to maintaining intestinal homeostasis and modulating the development and maturation of the immune system, which is pivotal to effectively protecting the host against pathogenic organisms (4). Thus, intestinal mucosal immunity continuously functions to maintain a delicate balance to the commensal organisms, to avoid unnecessary inflammation to exogenous antigens (including food allergens) or damage-associated self-antigens, and, very importantly, to prevent the invasion or dissemination of pathogenic organisms (5–9). Remarkably, the lamina propria layer of the small intestine retains the highest concentration of

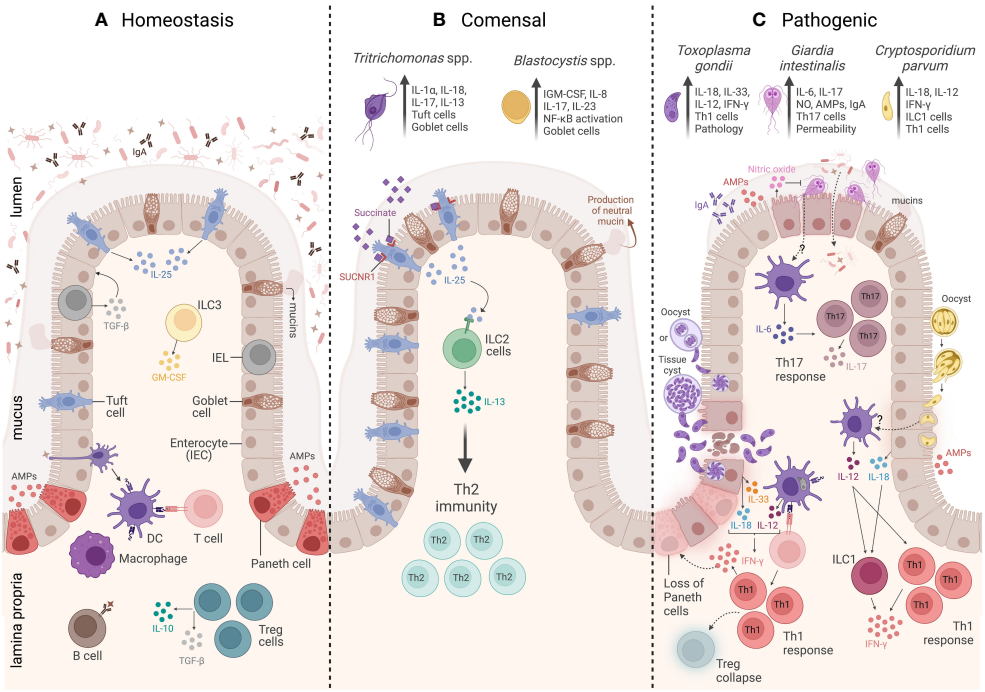

FIGURE 1
Overview of the intestinal mucosal immunity during homeostasis, commensal protozoa colonization, and pathogenic protozoa infection. **(A)** The homeostasis of a healthy intestine is maintained by several biochemical and cellular components that form a physical barrier, including a mucus layer that isolates the lumen from the epithelium and underlying the mucus. There is a group of specialized epithelial cells composed of enterocytes, goblet cells, and Paneth cells that secrete antimicrobial peptides (AMPs), tuft cells that constitutively express IL-25, and intraepithelial lymphocytes (IELs) that regulate epithelial growth through the secretion of TGF-β1. Underneath the epithelium is the lamina propria (LP), and the main cells involved in homeostasis are macrophages and dendritic cells (DCs) responsible for tolerance and T-cell activation. Innate lymphoid cell 3 (ILC3) is the main source of granulocyte–macrophage colony-stimulating factor (GM-CSF), while T regulatory cells (Tregs) are key to maintaining intestinal balance and tolerance through the secretion of IL-10 and TGF-β. **(B)** The commensal protozoa colonization is represented by two protists, *Tritrichomonas* ssp. and *Blastocystis* spp. In the small intestine, *Tritrichomonas* spp. secrete succinate molecules that bind in tuft cells SUCNR1 receptors, inducing these cells to release IL-25 resulting in ILC2 activation and IL-13 secretion, leading to Th2 response and goblet cells hyperplasia. Meanwhile, in the large intestine, *Tritrichomonas* spp. induce Th1 and Th17 immune responses by eliciting IL-1β, IL-18, and IL-17 cytokine release. *Blastocystis* colonization induces goblet cell hyperplasia, which leads to neutral mucin production and stimulates Th1/Th17 immune response, with IL-17 and IL-23 cytokine signatures. **(C)** The intestinal mucosal immunity to pathogenic protozoa infection is represented by three parasites: *Toxoplasma gondii*, *Giardia intestinalis*, and *Cryptosporidium parvum*. *T. gondii* infection is characterized by a strong Th1 immunity, tissue damage, and immunopathology. The immune response to this protozoan is characterized by high levels of IL-33- and IL-18-producing epithelial cells, as well as IL-12 production by DCs. All these three cytokines act together to induce $CD4^+$ T cells producing IFN-γ. The infection is also characterized by Treg collapse and a loss of Paneth cells due to the high levels of IFN-γ. *G. intestinalis* infection is characterized by a Th17 immunity, elicited by IL-6-producing DCs and IL-17-producing $CD4^+$ T cells. In addition, *G. intestinalis* increases intestinal epithelial permeability and microbial translocation, as well as enhances IgA, AMPs, nitric oxide (NO) levels, and mucin secretion. *C. parvum* infection is characterized by a Th1 immune response, which is associated with secretion of AMPs and IL-18 by epithelial cells, IL-12 by DCs, and IFN-γ production by both innate lymphoid cells 1 (ILC1) and $CD4^+$ T cells. Created with BioRender.com.

immune cells, which mediate host protection against infective agents and also promote bystander inflammation and pathology (1).

Anatomically, the intestinal mucosa is composed of an epithelium, which consists of a folded single layer of epithelial cells, connected by tight junctions, that is superimposed by a mucus layer (10). It is organized into crypts (invaginations into the underlying mesenchyme) and villi (projections into the lumen) (11). The cellular composition of the epithelium includes enterocytes, goblet cells, Paneth cells, M cells, tuft cells, enteroendocrine cells, and intraepithelial lymphocytes (IELs) (12–14). Underlying the epithelium is the lamina propria (LP), formed by structural elements like fibroblasts, fibrocytes, vascular endothelia cells, muscle cells, and immune cells like macrophages, mast cells, dendritic cells (DCs), plasma cells, and B and T cells, which are responsible for maintaining homeostasis, immune tolerance to commensals, and a defensive barrier against invasive pathogenic agents (1, 13). The epithelium thus represents both a physical barrier and an important site for the production of cytokines, chemokines, and antimicrobial peptides that protect the host against various infections and danger signals (Figure 1A).

Epithelial cells are comprised of a diverse array of specialized cell types, which include goblet cells (professional mucin-producing cells responsible for forming a dense mucus gel layer) that function to protect and maintain healthy epithelial function; tuft cells are immune sentinels and taste-chemosensory cells that constitutively express IL-25; Paneth cells are important sources of antimicrobial peptides (AMPs; defensins and lysozyme) and growth factors, which are associated with host protection against enteric infections, maintenance of crypt stem cell activity, and intestinal homeostasis by their ability to regulate the microbiome in the lumen (11, 15–19). Finally, the IELs, composed of γδ T cells and $CD8^+$ T cells (expressing the CD8α homodimer), are interspersed throughout the intestinal epithelial layer and regulate epithelial growth and homeostasis by their secretion of TGF-β1 (20). IELs do not require priming to respond since they are antigen-experienced T cells that release cytokines and kill target cells upon encountering specific antigens (21, 22).

In the lamina propria, macrophages, DCs, innate lymphoid cells (ILCs), natural killer (NK) cells, and T and B lymphocytes are the immune cells that work together to maintain intestinal mucosal homeostasis and barrier integrity. The myeloid compartment, represented by macrophages and DCs, is crucial for the maintenance of intestinal tolerance and the activation of T-cell immunity (23, 24). The recently described innate immune cell family of ILCs within the intestinal mucosa is comprised of three different groups, namely, ILC1, ILC2, and ILC3. Of note, ILC3s are critically involved in intestinal homeostasis and consist of the primary source of granulocyte–macrophage colony-stimulating factor (GM-CSF) (25–27). Intestinal microbes, by their ability to promote B-cell receptor (BCR) editing and the immunoglobulin diversification of resident B cells, also play an important role in the induction of tolerance against commensal antigens in the mucosa (28, 29). The intestinal mucosa is likewise home to the largest number of T cells in the body, comprised of a heterogeneous array of $CD4^+$ and $CD8^+$ T cells (29, 30). Most of these T cells in the mucosa possess an activated/memory phenotype, and their activation culminates in a rapid proliferative response (31). Among the most enriched T-cell populations in the gut are regulatory T cells (Tregs), which promote the maintenance of immune intestinal balance and tolerance, mainly by the secretion of IL-10 and TGF-β (32).

Understanding the orchestration and characteristics of the mucosal immune response during colonization by pathogenic or commensal organisms within the intestine may provide novel insights into the mechanisms underlying microbe-induced protection and/or immunopathology. Of note, most of our knowledge about the interactions between the host mucosal response and the gut microbiome, including the eukaryome, is dominated by studies focused on bacterial communities, helminth parasites, and intestinal viruses. Importantly, the impact of commensal and pathogenic eukaryotic protists that colonize the intestinal mucosa is less studied. In this review, we provide an overview of the current knowledge of protozoan-induced intestinal mucosal immunity, specifically, how commensal and pathogenic protozoa trigger the development and modulation of innate and adaptive immune responses in experimental models of infection and what the pathological consequences are for the host, if any.

Mucosal immunity to commensal intestinal protozoa

Investigating the host gastrointestinal immune response to the microbiome is an emerging field. Most studies are focused on host responses generated against enteric bacterial communities. However, recent advances in amplicon-based next-generation sequencing (NGS) and metagenomics analysis have allowed a more comprehensive identification and description of the diverse micro-eukaryotes living within the mammalian microbiota, such as the fungi and protozoa that comprise the eukaryome (33). Large and small intestine-colonizing eukaryotes are commonly associated with pathogenic organisms such as *Toxoplasma gondii*, *Giardia* spp., and *Cryptosporidium* spp., which are discussed further in the second part of this review. However, enteric non-pathogenic commensal protozoa have also been identified by their ability to alter the gut–bacteriome diversity and to prime the host-immune response (34). Currently, relatively few investigations have examined gastrointestinal tract (GIT) immunity generated by the presence of protozoa that exist as enteric commensals. In this

review, we specifically focus on *Blastocystis* spp. and *Tritrichomonas* spp. (Figure 1B).

Blastocystis spp.

As reviewed by Parija and Jeremiah, *Blastocystis* spp. were first described by Brittan and Swayne during the 1849 cholera epidemic in London. To this day, the taxonomic classification of this organism remains controversial in the scientific community (35). In the early 1900s, Alexeieff and Emile Brumpt classified *Blastocystis* as a harmless saprophytic yeast of the intestinal tract. However, in 1967, Zierdt *et al.* reclassified the organism as a protist based on its morphology and phenotypic properties (36). Thus, after various observations over the decades, *Blastocystis* is currently classified as an anaerobic, non-invasive protist that colonizes the mammalian intestinal tract and belongs to the Stramenopiles, a clade of protists that group with algae, diatoms, and water molds (37). It possesses a high genetic diversity and a vast host range, including livestock and humans (38). Epidemiological estimates suggest that more than 1 billion people worldwide are colonized with *Blastocystis* spp., varying at 0.5%–23.1% in developed countries versus 22.1%–100% in resource-limited countries (39–41). *Blastocystis* spp. is currently comprised of 28 subtypes (STs), 12 of which are reported to colonize the GIT of humans (ST1–ST8, ST10, ST12, ST14, and ST16), but among them, only ST1–ST4 account for more than 90% of the human infections reported globally, whereas ST5–ST8, ST10, ST12, ST14, and ST16 are rarely found in human stool (42–46). *Blastocystis* has been detected in the human enteric microbiota in both healthy and disease conditions by different methods such as real-time PCR, amplicon-based NGS, and metagenomics. Its presence within the gut microbiota is associated with a shift in bacterial composition (47). The association between *Blastocystis* and the shift in microbial diversity and composition, as well as its effects on the host response remain to be determined. Although reports of this parasite are becoming more common in human gut studies, the direct *Blastocystis* mucosal immune response in the murine model is still under-explored.

Whether *Blastocystis* is pathogenic under some circumstances, for example, during host immune suppression, remains unclear, largely because screening for the presence of other known microorganisms such as viruses, protozoa, or bacteria has not been systematically performed (48, 49). Some studies have described a positive correlation between *Blastocystis* colonization and conditions such as irritable bowel syndrome or inflammatory bowel disease in humans (50, 51), whereas the high prevalence of this protist observed in healthy human guts has fueled a separate debate on whether this parasite is non-pathogenic and a commensal of the human GIT. Indeed, a sample cohort of healthy humans in Ireland identified that 56% of individuals were positive for *Blastocystis* by 18S rRNA PCR (52).

In vitro models suggest that *Blastocystis* can regulate host immune responses. Colonic epithelial cells exposed to *Blastocystis* Nand II strain released IL-8 and GM-CSF cytokines (53), possibly triggered by *Blastocystis* cysteine proteases, which are released by the zoonotic isolate WR1 (ST4) and activate the NF-κB transcription factor (54). In addition, *Blastocystis* ST7 (B isolate) can evade the immune response by downregulating inducible nitric oxide synthase (iNOS) (55) and degrading human secretory immunoglobulin A (IgA) (56).

Iguchi *et al.* determined that *Blastocystis* RN94-9 strain (a subtype 4 isolate from a laboratory rat) is not inconspicuous. Although rats experimentally infected with ST4 RN94-9 cysts largely failed to display pathogenic markers during colonization, such as weight loss, diarrhea symptoms, mucosal epithelium lesions, or inflammatory cell infiltrates (57), they did, however, induce type 1 proinflammatory cytokines such as IFN-γ, IL-12, and TNF-α and cause a mild goblet cell hyperplasia that was associated with an increase in the production of neutral mucins (57). Goblet cells are essential cells for maintaining gastrointestinal homeostasis. They synthesize neutral and acidic mucins to produce and sustain the enteric mucus layer, an innate barrier against pathogens (58). Alterations in the neutral and acidic mucin ratio have also been detected in rats infected by the parasitic nematode *Nippostrongylus brasiliensis* (59). While this imbalance in the production of neutral versus acidic mucins is thought to change mucus layer charge, whether it influences parasite-mucosa attachment is less clear.

In addition to the activation of a Th1 cytokine signature, *Blastocystis* modulates the *in vivo* Th17 cytokine immune response. Th17 immunity is strongly correlated with the clearance of extracellular parasites, inflammation, and auto-immune diseases (60). IL-17, IL-23, and IL-22 cytokines have a prominent role in Th17 subset activation (61). BALB/C mice infected with *Blastocystis* spp. developed significant increases in their IL-17 and IL-23 levels in the intestinal mucosa (62). In summary, *Blastocystis* appears to modulate the enteric host immune response by inducing specific Th1/Th17 cytokine production. Hence, colonization by *Blastocystis* may generate a level of bystander inflammation that could conceivably protect mice from pathogenic infections or make them more susceptible to certain auto-immune conditions. Therefore, studies to understand the kinetics, level of, and persistence of cytokine induction during *Blastocystis* colonization are needed. In addition, the data thus far investigating *Blastocystis* colonization *in vivo* certainly argue for the necessity to screen for the presence of commensal parasites in the gut in experimental workflows in order to gain a better understanding of their role in gastrointestinal disease models.

Tritrichomonas spp.

Tritrichomonas spp. comprise a complex group of neglected gut-dwelling protists that have similar morphology but possess

extant diversity. Studies within this group of parasites are limited, largely because of a lack of high-quality reference genomes or methods for axenic culture. Closely related species such as *Tritrichomonas muris*, *Tritrichomonas musculus*, and *Tritrichomonas rainier* have been discovered recently as common commensal protists that establish persistent, long-lived infections that impact the microbiome and mucosal immune homeostasis within the intestinal tract of wild and laboratory mice (63). Colonization of mice with these protists shows little to no evidence of epithelium lesion, blood cell infiltration, or other detectable gut-related disease symptoms, e.g., diarrhea, weight loss, or lethargy. Colonized mice do experience, however, a mild goblet cell hyperplasia and display significant changes in their mucosal immunity, which protect them from GIT bacterial infections but increase their susceptibility to inflammatory bowel-like diseases (64, 65). These studies certainly highlight the importance of screening the microbiota of laboratory mice for *Tritrichomonas* commensal infection.

In the healthy gut under homeostatic conditions, immune sentinels and taste-chemosensory cells called tuft cells are commonly found in low numbers within the epithelia of the small and large intestine in mice. However, in response to various parasitic infections, tuft cells are known to expand exponentially. For example, enteric non-pathogenic commensal protists, including *Tritrichomonas* spp., and pathogens such as nematodes (e.g., *N. brasiliensis*, *Heligmosomoides polygyrus*, and *Trichinella spiralis*) and trematodes (e.g., *Echinostoma caproni*) cause tuft cell activation and expansion, triggering mainly type 2 host immunity involving ILC2s (66–68).

Howitt *et al.* first described markedly different intestinal tuft cell numbers between specific pathogen-free BIH mice bred "in-house" versus BIH mice purchased from the Jackson Laboratory (JAX). When cecal contents from BIH mice were fed to the JAX mice, intestinal tuft cells proliferated to a level identified in the bred "in-house" BIH mice, and the transmissible component responsible for the phenotypic change was a single-celled protist that they referred to as *T. muris* (65). Two other contemporaneous studies likewise concluded that this and a related gut-dwelling commensal protist referred to as *T. musculus* alter immune cell homeostasis within the gut microenvironment, conferring the protection of colonized mice from mucosal bacterial infections at the expense of increasing their susceptibility to colitis and colorectal tumors (60, 67).

The Howitt study showed that shortly after *T. muris* colonization, tuft cells release the cytokine IL-25 and promote ILC2 activation and the secretion of IL-13, resulting in goblet cell hyperplasia and skewing the immune potential of the gut toward a Th2 response (65). Two years later, Nadjsombati *et al.* showed that the related parabasalid *T. rainier* also induced tuft cell expansion, ILC2 activation, and goblet cell hyperplasia in the small intestine of laboratory mice, similar to *T. muris* (69). Their work established that succinate secreted by *T. rainier* is a pivotal ligand sensed by the succinate receptor (SUCNR1) expressed on tuft cells and that this was sufficient to initiate a Th2-biased immune response in the small intestine (69). Succinate is a metabolite produced by hydrogenosomes, a mitochondria-like organelle that synthesizes ATP in anaerobic protists, including *Tritrichomonas* spp (70). This remarkable finding revealed for the first time that a metabolite unique to a eukaryotic microbe was able to directly activate type 2 immune cell responses *via* a tuft cell-ILC2 sensing and activation circuit (19).

Paradoxically enteric *Tritrichomonas* spp. infection also activates Th1 and Th17 immune cells and their signature cytokines during mouse colonization, in addition to initiating Th2-biased immune responses in the small intestine. In the large intestine specifically, *T. muris* stimulates a Th1-biased pro-inflammatory response, increasing IL-12/IL-23p40 and IFN-γ proteins to levels that exacerbate colitis in $Rag1^{-/-}$ mice post-adoptive T-cell transfer (71). Chudnovskiy *et al.* also demonstrated a $CD45^{+}$ hematopoietic cell expansion and the secretion of high levels of IgA in the large intestine of mice colonized with *T. musculus* (*T.mu*), a *T. muris*-related species (64). Like *T. muris*, *T.mu* also induced mild goblet cell hyperplasia and an exacerbated inflammatory response in colitis and tumorigenesis models. Within the cecal epithelium, high levels of IL-18 were released, the result of activating an ASC-dependent inflammasome, and this was important for the induction of colonic Th1 and Th17 immune responses in *T.mu*-colonized mice (64). The altered inflammatory state driven by the IL-18 release within the colonic epithelium was sufficient to protect mice from *Salmonella typhimurium* enterocolitis and established that the presence of *T.mu* significantly increased anti-bacterial defenses within the enteric mucosa (64). Recent work has identified that *T.mu* activates both the NLRP1B and NLRP3 inflammasomes and also causes a dramatic increase in luminal extracellular ATP levels (72). *T.mu* colonization likewise triggered IL-13 by ILC2s in the mucosa, as previously reported by Howitt *et al.* using *T. muris* (65). It also altered host glucose homeostasis by increasing gluconeogenesis, as well as increasing free choline production in the gut of infected hosts. The parasite metabolite that triggered this pathway is unknown, but there is evidence for a succinate-independent process (73). Altogether, the data described here emphasize the importance of investigating commensal protists in the gut microbiota in order to devise new therapeutic interventions to control inflammatory, pathogenic, and/or metabolic diseases.

Intestinal mucosal immunity to pathogenic protozoa

A healthy and functional intestinal mucosa is both plastic and dynamic, which is necessary to maintain immune homeostasis and protect hosts from invading pathogenic

organisms. However, dysregulated immune responses that result in chronic diseases within the gut epithelia have negative consequences for the host, including intestinal damage and a failure to control microbial pathogens (3). The following section summarizes the current knowledge on the intestinal mucosal immune response to the pathogenic protozoan parasites *T. gondii*, *Giardia intestinalis*, and *Cryptosporidium parvum* and describes the most relevant epithelial and immune cell populations involved in such responses (Figure 1C). Although these are all human parasites and their associated diseases are extremely relevant within the human population globally, our focus in this review is to describe the major features of the mucosal immune response triggered by these intestinal protozoa in experimental animal models to better understand the biology of their parasitism and highlight the parasite and host factors that specifically influence their pathogenicity.

Toxoplasma gondii

T. gondii is an intracellular protozoan parasite capable of infecting essentially any warm-blooded animal. It is highly prevalent worldwide, with about 1/3 of the human population chronically infected with *T. gondii* parasites (74). The parasite is a leading cause of infectious blindness and is considered a life-threatening disease among the immunocompromised. It is also capable of causing abortion or birth defects during congenital infection (75, 76). *Toxoplasma* transmission occurs by oral ingestion of infectious tissue cysts or oocysts; hence, intestinal mucosal immunity constitutes the first line of defense and is one of the most important barriers against *T. gondii* (13, 77, 78). In the small intestine, *T. gondii* differentiates into tachyzoites, the rapidly replicating form of the parasite, which ultimately disseminates infection beyond the intestinal mucosa, colonizing other tissues, such as skeletal muscle and the central nervous system, before it differentiates into the bradyzoite form, which establishes latent infection in the form of tissue cysts (13). When in the gut, *Toxoplasma* alters the mucosal homeostatic balance and induces a cascade of immunological events involving components of both innate and adaptive immunity, characterized by a polarized Th1 immune response that induces high levels of IFN-γ and a collapse of Tregs (13, 79). The heightened inflammatory state has been shown to cause a Crohn's disease-like enteritis, the result of an IFN-γ-mediated acute ileitis that occurs in some inbred mice (77, 80–83). Type 1 immunity is normally associated with host protection and is necessary for *T. gondii* clearance. However, in some *Toxoplasma* murine infection models, a dysregulated Th1 response can occur, which causes irreversible pathological alterations and inflammatory responses that promote severe tissue damage and host death (82–84). Here, we describe the mucosal-associated immune cell types that are induced during *T. gondii* infection in the gut with reference to well-described parasite factors that regulate the development of the *T. gondii*-associated mucosal immune response.

Gut epithelial and lamina propria cells are key players in host defense against *Toxoplasma* infection in the intestine. The gut epithelium is an important physical barrier and functions as the first line of innate defense against the parasite. In response to oral *Toxoplasma* infection, the gut epithelium releases alarmins IL-18 and IL-33 (both IL-1 family members), which synergize with IL-12 to promote protective IFN-γ-mediated immune responses that regulate *T. gondii* infection within the ileum. The release of IL-18 is caspase-1/11, ASC, and inflammasome sensor NLRP3- and NLRP1b-dependent (85). IL-18 plays a pivotal role in limiting parasite replication, as mice deficient in IL-18 or IL-18R experience 20–100-fold higher parasite loads and die acutely, compared to infected wild-type mice with the same genetic background (85, 86). It has also been reported that *T. gondii* actively crosses the gut epithelial barrier by invading, replicating, and lysing epithelial cells (78, 87). This process ruptures the gut epithelium and releases the damage-associated molecular pattern (DAMP) cytokine IL-33, which also synergizes with IL-12 to promote ILC1 production of IFN-γ and CCR2-dependent recruitment of inflammatory monocytes required for resistance to *T. gondii* (88). *T. gondii* tachyzoites are also released into the lamina propria compartment. Such collateral damage facilitates the translocation of bacteria across the gut epithelium, which exacerbates the inflammatory response and causes serious gut pathology (78, 89–92).

The presence of IFN-γ driven by *T. gondii* infection is also responsible for the rapid loss of Paneth cells within the host mucosa and results in a myriad of pleiotropic effects. Paneth cells are epithelial cells that secrete a suite of antimicrobial proteins and peptides in order to sustain mucosal homeostasis (15). In the presence of *Toxoplasma*, alterations in both membrane and mitochondrial integrity occur in Paneth, cells which activate an mTORC1-dependent cell death pathway (93–95). The loss of Paneth cells is also partially associated with the activation of TLR11 signaling in the gut (93). Likewise, IELs, another epithelial cell type comprised mainly of CD8α T cells, play a critical role in the initiation of tissue damage and immunopathology in response to *Toxoplasma* infection. The progression of pathological inflammation and necrosis is associated with the expansion of $CCR2^{hi}CD103^{+}$ IELs in the epithelium, which are principally induced by the presence of the CCL2 chemokine. Interestingly, $CCR2^{-/-}$ mice experience diminished intestinal inflammation but had higher mortality rates, due largely to a failure to regulate *Toxoplasma* growth (96). Hence, IELs perform a critical function. Failure to regulate them precisely represents a double-edged sword: IELs secrete anti-inflammatory mediators, like TGF-β, to regulate mucosal inflammation in the GIT but can also expand to produce high levels of IFN-γ, which drives the immunopathology in response to *Toxoplasma* infection (77).

After crossing the epithelial barrier, parasites reach the LP, where immune cells are abundantly present. In the LP, innate immune cells, including inflammatory monocytes, dendritic cells, and NK cells, are critical players that both initiate and regulate the immune response against *T. gondii* infection (76). The myeloid compartment, specifically macrophages and dendritic cells, is responsible for sensing parasite antigens (pathogen-associated molecular patterns (PAMPs)) and rapidly respond by secreting IL-12, one of the major cytokines released in response to *Toxoplasma* infection. IL-12 principally drives the marked IFN-γ production by NK and T cells (97, 98). Among innate immune cells of non-myeloid origin, the role of ILCs during *T. gondii* infection is not well defined, so it remains unclear precisely how they contribute to the induction or regulation of mucosal immunity against *Toxoplasma*. Although it is known that ILC1 cells can secrete IFN-γ, the contribution of this cell type to the total aggregated IFN-γ levels during *Toxoplasma* infection is likely less relevant than that of $CD4^+$ T cells. Indeed, López-Yglesias *et al.* demonstrated that in the absence of ILC1, $Tbx21^{-/-}$ mice were still able to generate a robust IFN-γ response driven by $CD4^+$ T cells (94). Of note, Wagage *et al.* suggested that $ROR\gamma t^+$ ILC3 cells limit T-cell responses and pathology during *T. gondii* infection; however, they showed that ILC3 frequency is significantly decreased in the presence of the parasite (99). More studies on ILCs are required to fully understand their role in oral *T. gondii* infection models. Likewise, the role of neutrophils, another innate immune cell of myeloid origin, has not been systematically studied, so its role in contributing to mucosal immunity during *Toxoplasma* infection is not clear. Neutrophils rapidly migrate into the lamina propria during acute infection and can be detected within 3 days post-oral inoculation of *T. gondii* cysts (100, 101). Coombes *et al.* reported that tachyzoites preferentially infect infiltrating neutrophils, which function as reservoirs for *T. gondii* dissemination to other host tissues (102). Further studies are necessary to define the role of neutrophils during *Toxoplasma* intestinal infection.

One of the most important and well-studied cell types responding to *T. gondii* infection is DCs. These cells are the main source of IL-12 production and are central for the activation of T cells during both intraperitoneal and oral *T. gondii* infection. Several studies previously demonstrated that *T. gondii* proteins can directly activate Toll-like receptors (TLRs) to induce IL-12 production by DCs and macrophages. These include Micronemes 1 and 4 (TLR2/TLR4 agonists) and Profilin (TLR11/TLR12 agonists) (103–107). Although most of these studies were carried out using intraperitoneal infection models, it is known that profilin and TLR11 interactions also activate the intestinal innate immune response during oral infection with *T. gondii*. Benson *et al.* showed that mice deficient in TRL11 expression are more susceptible to *Toxoplasma* infection, the result of a marked reduction in IL-12 secretion and a partial defect in the generation of Th1 cells (108). In addition, recent studies have reported that IL-12—released during *Toxoplasma* infection—arises mainly from uninfected lamina propria DCs ($CD11b^-CD103^+$) rather than infected ones (109–111). It has been suggested that these cells respond to soluble parasite factors secreted prior to parasite invasion or parasite proteins that have been actively injected into DCs by *T. gondii* (109–111). Other parasite factors that regulate mucosal immunity include the Type II dense granule protein 15 ($GRA15_{II}$), which activates NF-κB within innate immune infected cells and results in elevated IL-12 production, lower parasite burden, and less intestinal inflammation. This event was reported to occur only when the Type I rhoptry kinase 16 ($ROP16_I$), another parasite factor that is involved in STAT3, STAT5, and STAT6 activation, was heterologously expressed within *T. gondii* Type II strains (112, 113). Hence, the combined expression of $GRA15_{II}$ and $ROP16_I$ in a Type II background strain conferred host resistance to acute oral infection by *T. gondii*. Finally, GRA24 is also released into the host cell cytoplasm to induce IL-12 production by triggering the activation of host mitogen-activated protein kinase p38 (p38 MAPK), which subsequently results in the transcription of the IL-12 gene (114–117). Additionally, different studies reported that there is a large CCR2-dependent influx of inflammatory monocytes into the lamina propria compartment during *Toxoplasma* infection, and IFN-γ-activated inflammatory monocytes are associated with the control of parasite replication and host resistance (111, 118). These cells are capable of producing IL-12 in the gut during infection, and this helps in the recruitment of protective Th1 cells in a CXCR3-dependent manner (119). Moreover, it is also reported that inflammatory monocytes are critical for *T. gondii* clearance by IFN-γ-dependent activation of iNOS/NOS2, the release of NO, and the activation of IRGs (immunity-related GTPases), which destroy the parasitophorous vacuole membrane (PVM) to limit parasite replication (92, 120, 121).

As mentioned before, *T. gondii* infection induces a very strong and polarized Th1 immune response, associated with a high frequency of IFN-γ-producing $CD4^+$ T cells in murine models. Although Th1 immunity is required to control parasite replication, by inducing such a strong inflammatory response, in some mouse genetic backgrounds, it also drives a severe, and often lethal, intestinal immunopathology (81, 93, 122). López-Yglesias *et al.* demonstrated that even in the absence of T-bet, the transcriptional factor signature for Th1 cells, the IFN-γ-producing $CD4^+$ T-cell response, is still markedly increased in $Tbx21^{-/-}$ mice, suggesting that the immunopathogenesis during infection can be driven by a T-bet-independent pathway (94). Additionally, the *T. gondii*-Th1-induced tissue damage in the intestinal mucosa is associated with the collapse of resident Tregs and a subsequent reduction of IL-10 levels during infection (79). This phenomenon is associated with the conversion of Tregs into $Tbet^+$ IFN-γ-producing cells and favors the development of a Th1-driven Crohn's disease-like

immunopathology (79, 123). Finally, Rachinel *et al.* suggested that the acute ileitis driven by *T. gondii* is associated with the expression of the parasite immunodominant antigen SAG1 (surface antigen 1), which elicits a $CD4^+$ T cell-dependent lethal inflammatory process in the oral B6 mouse model (124). Although Th1 responses dominate during *T. gondii* infection, a few studies suggest that IL-17A- and IL-22-secreting Th17 cells also play an important role in maintaining persistent levels of antimicrobial peptide production, given that mice deficient in class I-restricted T cell-associated molecule (CRTAM) expression on T cells failed to control *T. gondii*-driven immunopathology and microbial translocation (125, 126).

Among all the intestinal pathogenic protozoa parasites that will be discussed in this review, it is notable that the area of study regarding *T. gondii* biology has evolved dramatically during the past few years. In addition, advances in gene editing tools and experimental models are continually contributing to our understanding of important host–parasite interactions that impact host immune responses against *T. gondii*. However, the role of many parasite factors and their interaction impacting the development of host intestinal immunity remains unknown. Ultimately, further studies are required to comprehensively elucidate the underpinnings of the protective immune response that can be elicited to revert immunopathology and confer a host-protective effect by reducing or preventing *T. gondii*-driven acute ileitis.

Giardia intestinalis

The intestinal extracellular protozoan parasite *G. intestinalis* (syn. *Giardia duodenalis* and *Giardia lamblia*) is highly prevalent worldwide and can infect all mammals, including humans, domestic animals, and wildlife. Giardiasis is considered one of the most prevalent enteric diseases caused by a protozoan parasite, with estimates ranging from 2%–5% to 20%–30% of *Giardia*-infected people in high and low-middle-income countries, respectively. Approximately 280 million new cases are documented every year worldwide (127–129). Immunocompetent individuals typically resolve the infection spontaneously within a few weeks after exposure, and some people never develop any symptoms (130, 131). However, susceptible individuals often exhibit a myriad of different gastrointestinal symptoms, including abdominal cramps, nausea, and diarrhea (132). These clinical symptoms are frequently associated with the development of malabsorption syndrome and with children's development and growth impairment, especially in chronic and recurrent *Giardia* infections (133–135). Recent data suggest that giardiasis is one of the four main contributors to stunting in children (135–137). Transmission of *Giardia* occurs through the oral–fecal route with the ingestion of parasite cysts present in contaminated water or food (138), highlighting the importance of the intestinal mucosal immune response triggered by *Giardia* infection, which will be discussed here.

Within the gastrointestinal environment, *Giardia* cysts release trophozoites, the parasite form that adheres to the epithelial monolayer to colonize the small intestine, especially the duodenum. Trophozoites attach to the microvilli in the epithelium to replicate and complete their life cycle, but this alters mucosal homeostasis and induces several host responses. In mice, *Giardia* infection is mainly characterized by a protective Th17 immune response, with high levels of IgA secretion, and the presence of IL-17 is associated with parasite clearance (139, 140).

The intestinal epithelium functions as a physical barrier to protect from invading pathogens reaching the deeper layers of the mucosa. Although *Giardia* trophozoites do not invade the mucosa or submucosa layers in immunocompetent organisms, many studies suggest that *Giardia* trophozoites attach to the intestinal epithelial cells (IECs) and rupture the epithelial barrier by disrupting tight junction proteins within the IECs, such as claudins and occludin, which increases intestinal permeability, facilitating the entry and spread of enteric pathogens (141–143). In addition to functioning as a physical barrier to avoid parasite invasion in the lamina propria, the intestinal epithelial layer is responsible for producing various chemicals, such as nitric oxide (NO), and antimicrobial peptides that inhibit *Giardia* replication in the epithelium (144). However, *Giardia*-produced arginine deiminase (ADI) is thought to reduce the availability of arginine in the gut, which negatively affects NO production by enterocytes (144). In addition, Stadelmann *et al.* reported that arginine consumption by *Giardia* is also associated with decreased proliferation of intestinal epithelial cells during infection (145). Epithelial Paneth cells produce and release α-defensins, an AMP that, in combination with NO from nitric oxide synthase (NOS2), acts to control *Giardia* burden and eliminate infection (146). *Giardia* infection also upregulates mucus production during intestinal infection (147–149).

Giardia infection results in the rupture of the epithelial barrier, and this event is associated with the release of chemokines that recruit immune cells required for the protective response against *Giardia*. The epithelial disruption also promotes microbial translocation and the release of parasite antigens within the lamina propria, which induces the activation of recruited and resident myeloid cells (140, 150–152). However, studies regarding the specific role of macrophages and dendritic cells during *Giardia* infection in mice are sparse in the literature. It is known that DCs are one of the main sources of IL-6, and in the absence of this cytokine, IL-6 knockout mice fail to control *Giardia* replication during *in vivo* infections (153–155). It is reported that *Giardia* can induce a cytokine profile that is less inflammatory, and it seems that the reduction of proinflammatory cytokines is mediated by the activation of TLR2 signaling in DCs and macrophages (156, 157). Paradoxically, infection in mice that lack TLR2 is associated

with reduced parasite burden and less *Giardia*-associated pathology (156). Further studies are necessary to understand the specific role of myeloid cells during *Giardia in vivo* infection.

Giardia induces the recruitment of mast cells into the small intestine lamina propria, and at the site of infection, these cells degranulate and release histamines and protease-1 (MMCP-1) (158, 159). Li *et al.* described that MMCP-1 interacts with cholecystokinin (CCK), and this interaction results in more intestinal contractility, which is thought to be associated with cramps, one of the most common symptoms noted by infected people (128, 159). The literature suggests that mast cells are recruited *via* activation of the complement lectin pathway since mice lacking mannose-binding lectin 2 (MBL2) and complement factor 3a receptor (C3aR) expression are impaired in mast cell recruitment during *Giardia* infection (160). Additionally, the expression of MBL2 in the intestinal mucosa is dependent on *Giardia*-induced IL-17 (147).

As mentioned before, *Giardia* infection induces the differentiation and expansion of IL-17-producing $CD4^+$ T cells, which confer a host-protective effect by limiting parasite replication. It is reported that the control of infection is dependent on T cells but independent of Th1 and Th2 immunity (139, 140, 161). However, as is the case for many other pathogens, a protective immune response that limits the *Giardia* burden may also generate significant pathology. Known sequelae associated with *Giardia* infection in mice are accelerated intestinal transit contributing to parasite-induced diarrhea, muscle hypercontractility, mast cell activation, and cramping (158, 159, 162, 163). Lastly, activated $CD8^+$ T effector cells ($CD44^{hi}$ and $CD69^{hi}$) mediate tissue damage and microvillous shortening, which is responsible for disaccharidase enzyme (sucrase and lactase) deficiency and malabsorption of electrolytes, nutrients, and water (156, 164). The mechanism of $CD8^+$ T-cell activation during *Giardia* infection, however, remains unclear but is thought to be influenced by the intestinal microbiota (156).

While there has been significant progress in understanding the host–parasite interaction and the immune response induced by *Giardia*, further studies are needed to determine which parasite factors specifically trigger innate immune activation, as well as the signals implicated in Th17 activation within the intestinal mucosa, and how $CD8^+$ T cells become activated to induce pathology. Finally, new studies are required to better understand the nature of the protective immune response induced by *Giardia*, which is associated with a reduced risk of developing a severe diarrheal disease in children, and whether *Giardia* parasitism may be relevant or harmful in the context of a co-infection with another enteric pathogen.

Cryptosporidium parvum

Cryptosporidium spp. are epicellular protozoan parasites that colonize the gastrointestinal tract of mammals. The species *C. parvum* and *Cryptosporidium hominis* cause the majority of human infections globally (165, 166). In immunocompetent individuals, cryptosporidiosis is usually a self-limiting infection, with mild (abdominal pain and diarrhea) or absent symptoms, but this parasite can cause serious diseases in immunodeficient patients leading to severe, life-threatening diarrhea (166, 167). The enteric disease is also the second leading cause of severe diarrheal illness in children under 5 years old, with an estimated 60,000 deaths per year worldwide (168). In addition, it is responsible for affecting the growth and development of children. The limited availability of therapeutic treatments and the total lack of a vaccine represent a challenge for disease prevention (168). Parasite transmission occurs through the oral–fecal route upon the ingestion of infectious oocysts (169). Among the three pathogenic protozoa discussed in this review, *Cryptosporidium* infection and its associated mucosal immunity are much less explored by the scientific community, mainly because of a lack of experimental tools, including the ability to readily cultivate the parasite life cycle in the laboratory and the limited availability of animal models. Nevertheless, recent advances adapting *Cryptosporidium* to mice are facilitating new insight and permitting investigation of the intestinal mucosal immune response during *in vivo* infection.

Within the intestinal lumen, released sporozoites adhere to the epithelial plasmalemma to form a parasitophorous vacuole, a compartment isolated from both the lumen and host cell cytoplasm where parasites replicate and promote colonization of the small intestine. Cryptosporidiosis is characterized by crypt hyperplasia, villous atrophy, and diffuse shortening or loss of brush border microvilli, which results in malabsorption, dehydration, and diarrhea (166, 167). *Cryptosporidium* initiates an inflammatory process by activating epithelial cells (IECs) and inducing the secretion of "alarmin" cytokines (IL-8, TNF-α, and IL-1β) and chemokines (CCL2, CCL5, CXCL1, CXCL8, CXCL9, CXCL10, etc.) (170, 171). This event triggers the recruitment of effector immune cells to the site of infection and inhibits parasite adhesion to the mucosal epithelium (169–172). Various other experimental studies have concluded that production of β-defensin antimicrobial peptides, as well as cytokines, chemokines, and prostaglandin E2 by IECs, occurs *via* activation of the TLR2/TLR4/NF-κB signaling pathway during *Cryptosporidium* infection (171, 173, 174). Moreover, NF-κB activation induces the expression of anti-apoptotic factors that prolongs the life of IECs, promoting parasite replication and the propagation of the infection (175, 176).

The secretion of chemokines by *Cryptosporidium*-activated IECs is responsible for the recruitment of inflammatory monocytes, macrophages, and NK cells to the site of infection, and the presence of local IL-18 and IL-12 induces a synergistic activation of macrophages and NK cells to secrete high levels of IFN-γ in infected neonatal mice (170, 177). Depletion of NK cells was associated with an exacerbated infection in neonatal mice (178). Furthermore, Choudhry *et al.* reported that the

neutralization of IL-18 during *Cryptosporidium* infection, after *in vivo* treatment with anti-IL-18 antibodies, resulted in decreased IFN-γ expression in $Rag2^{-/-}\gamma c^{-/-}$ mice (177). Different studies using transgenic mice have shown that deficiencies in either Th1 cytokines, such as IL-12, IL-18, and IFN-γ, or inflammasome components (caspase-1) are associated with a more susceptible or sometimes lethal phenotype to *Cryptosporidium* infection (179–183). Additionally, a recent study has demonstrated that *Cryptosporidium* is recognized by the inflammasome sensor NOD-like receptor family pyrin domain containing 6 (NLRP6), which activates enterocytes to release bioactive IL-18 and is required for parasite control (183). Finally, the synergistic secretion of IL-18 and IL-12 by activated enterocytes stimulates ILCs to produce IFN-γ, and this event promotes parasite control by enterocytes (184). Thus, the combination of IL-12, IL-18, and IFN-γ as well as inflammasome activation seem to be associated with a protective mechanism against infection.

Given the fact that IFN-γ suppresses *Cryptosporidium* infection and controls parasite replication, it has been described that $CD4^+$ T cells are also essential for parasite elimination and the establishment of an effective immune response following infection (166). Many studies have reported that neutralization of IFN-γ in $Rag2^{-/-}$ or SCID mice was associated with increased *Cryptosporidium* burden, persistent diarrhea, and progress of intestinal pathology, sometimes leading to mouse death (179, 185–189). Intriguingly, McDonald *et al.* showed evidence that during early *Cryptosporidium* infection, mice secreted intestinal IL-4. They showed that susceptibility to infection was increased and associated with higher production of oocysts when mice were treated with anti-IL-4 antibodies. Their data suggest that early IL-4 can promote a protective, Th1-mediated mucosal immune response that inhibits parasite development (190). The concomitant neutralization of IL-4 and IL-5 was also shown to increase oocyst shedding in infected mice (191). Lastly, the role of $CD8^+$ T cells and B cells during *Cryptosporidium* infection is less clear. Although it has been reported that they expand during infection, whether they are essential for the clearance of infection remains enigmatic (192, 193). Additionally, while parasite-specific IgM and IgG levels have been shown to increase during infection, they do not prevent the host from secondary infection, although specific antibodies are known to reduce oocyst shedding in a reinfection event (194, 195).

Despite the difficulties of conducting experimental studies in the *Cryptosporidium* field, knowledge is quickly evolving given the development of new technologies and lab strategies to improve parasite culture, life cycle, and experimental models. These new techniques are allowing previously unanswered questions about the biology of *Cryptosporidium* parasitism to be explored, including how parasite proteins can be delivered directly to the cytosol of infected host cells (196). Still, many questions remain unanswered, such as what parasite factors are recognized by the host cells that initiate the innate mucosal immune response or what extracellular or intracellular receptors are involved in parasite recognition.

Microbial dysbiosis: A common feature between commensal and pathogenic protozoa

The identification of the gut eukaryome, including some intestinal protozoa discussed in this review, has allowed scientists not only to characterize their interaction with the mammalian host and their capacity to re-shape intestinal immunity but also to investigate their association with a large group of bacterial communities that inhabit the mammalian gut and specifically how these interactions might alter host gut homeostasis and promote disease pathology (33, 64).

Apart from the ability to sense intestinal immunity, all enteric protozoa discussed in this review share a mutual capacity to alter the host gut microbiome. These microeukaryotes, independent of their pathogenic or commensal status, alter the diversity of the microbiome during colonization of the intestine, which can alter mucosal immune homeostasis and promote diseases (34). For instance, recent studies suggest that colonization with *Tritrichomonas* is associated with decreased microbial diversity (197), whereas colonization with *Blastocystis* increases microbial diversity and is associated with the expansion of Clostridia and depletion of Enterobacteriaceae (198), which reduces inflammation by promoting a more healthy gut microbiota. However, some enteric protozoa, such as *Toxoplasma* (91, 92, 199), *Giardia* (200–202), and *Cryptosporidium* (203, 204), promote a shift in microbial community structure associated with dysbiosis, which exacerbates diseases and facilitates bacterial translocation (205). For example, *T. gondii* causes a profound expansion of Enterobacteriaceae (especially *Escherichia coli*), resulting in dysbiosis and increased immune-driven pathology in C57BL/6J mice (92). Additionally, Wang *et al.* showed that host-derived nitrate generated by the activation of the immune response against *T. gondii* promoted the overgrowth of Enterobacteriaceae. This occurs due to the activation of the IFN-γ/STAT1/iNOS pathway in macrophages, which is the same pathway associated with parasite control during infection (92). Indeed, it has been shown that the impaired activation of macrophages, by the absence of STAT1 signaling or deficiency in CCR2 expression or by blocking TNF-α with neutralizing antibodies, can induce a marked reduction of Enterobacteriaceae expansion (90). Interestingly, the influence of enteric protozoa

on the gut microbiome may also be regulated by host genetic background, because BALB/cJ mice infected with *T. gondii* do not develop enteric dysbiosis, nor do they promote nitrate-dependent overgrowth of Enterobacteriaceae, and they do not develop a dysregulated mucosal immune response and lethal ileitis, as seen in C57BL/6J mice (206–208).

Nevertheless, the common ability of intestinal commensal and pathogenic protozoa to induce microbial shifts during colonization results in different microbiomes and disease profiles, which can help to explain the divergence in the type of mucosal immune response that develops and whether a protozoan parasite is considered a commensal or a pathogen. Finally, the type of microbial shift that occurs and the mucosal immune response that develops not only help to explain how variations in disease can occur but may also determine whether the parasite is cleared, establishes a persistent infection, and/or is transmissible.

What makes a protist commensal or pathogenic?

The distinction between a host-associated commensal (defined as a microbe eating at the same table) and a pathogen (a microbe that causes harm) is often obscure. Depending on the context, some commensals can cause diseases (i.e., *Blastocystis*), whereas some pathogens can persist without causing overt diseases (i.e., *Cryptosporidium* and *Giardia*). The late Stanley Falkow perhaps said it best, that some microbes, he termed "commensal pathogens", can persist as natural members of the indigenous flora but possess an innate ability to cross anatomical barriers, invade tissues, or breach host defenses that ordinarily limit the survival or replication of other microbes or commensals (209). Perhaps the central question is rather how to limit these invasive properties in order to reduce disease potential. As mentioned above, many factors are likely to influence the relative pathogenicity of intestinal microeukaryotes, including 1) microbial shifts that expand or decrease specific classes of bacteria (taxa) that activate or regulate the immune response, 2) the host species and its genetic background, 3) the presence of microbe-associated molecular patterns (MAMPs) that exist as virulence factors that induce inflammation and/or pathology, or 4) the induction of chronic inflammation or granulomatous reactions to contain the infection. The microbial shift induced by pathogenic protozoa often differs from that induced by commensal protozoa. The molecular basis for this is varied; for example, the diversity of host or bacterial-associated metabolites can skew the immune response to either a regulatory profile or a more inflammatory one (210–213). Moreover, the bioproducts from enteric protozoa, such MAMPs, especially present in pathogenic microbes, can activate pathological aspects of the host immune response, and the inability to regulate this response may cause diseases (3). For instance, many bacteria can be both commensal and pathogenic, such as *E. coli*, a gram-negative versatile bacterium that is a commensal organism of the healthy gut microbiome but, through the acquisition of virulence genes by horizontal gene transfer (HGT) and/or accumulation of mutations in the genome, can convert from a commensal bacterium to a pathogenic one capable of causing a wide range of extraintestinal diseases (214–219). Similar mechanisms are known to occur in other organisms, where many species or strains initially not pathogenic evolved as pathogens due to the acquisition of virulence genes or the expansion of multi-gene families and/or epigenetic regulation mechanisms that promote invasive properties (220, 221). In addition, pathogenicity could also be associated with the relative abundance of specific microbes in the gastrointestinal tract, where their uncontrolled growth promotes the transition of microeukaryotes into pathogenic ones no longer controlled by host immunity (222). Thus, many protists can be considered both commensal and pathogenic; what determines their relative pathogenicity is a combination of different factors that together combine to determine their virulence potential.

Future perspectives

Throughout this review, it became clear and fascinating that studies focused on the investigation of intestinal protozoan biology have progressed extensively over the last decade. The discovery of new commensal protozoa and how they can reshape the host intestinal immune response has been extremely important in understanding the alteration of gut–bacteriome diversity and how this can affect the priming of mucosal immunity in general, which ultimately impacts (or defines) how the host will respond to gastrointestinal insults, such as parasitic infections or inflammatory diseases. Nevertheless, further studies are required to characterize these mechanisms in more detail and shed important insight specifically on how intestinal protozoa reshape intestinal immune potential in naïve hosts. Along with the discoveries about commensal protozoa, the advances in gene editing tools and experimental models are continually contributing to our understanding of the host–parasite interaction that impacts host mucosal immune responses against pathogenic protists. However, the precise parasite factors and how they trigger host intestinal immunity remain understudied. This review has discussed our current understanding of the intestinal mucosal immune homeostatic landscape and how it shifts in response to colonization by commensal or pathogenic protozoa. We believe that future work in this area will continue to shed important perspectives on the mechanisms underlying microbe-induced protection

and/or immunopathology and will identify new biomarkers for therapeutic intervention to clear parasites and control disease.

Author contributions

Conceptualization: AS-S. Funding acquisition: MG. Writing – original draft: AS-S and EA-F. Writing – review and editing: AS-S and MG. All authors contributed to the article, read, and approved the submitted version.

Funding

This study was supported by the Division of Intramural Research (DIR), National Institute of Allergy and Infectious Diseases (NIAID), NIH.

Conflict of interest

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

The handling editor declared a shared affiliation with the authors at the time of review.

Publisher's note

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